Joana Nata Albino Guerreiro
Degree in Biochemistry

Exploring the Anti-diabetic Effect of White Wine

Dissertation to obtain the Master Degree in Biochemistry for Health

Supervisor: Ana Teresa Serra, PhD, iBET/ITQB-UNL

November, 2016

Joana Nata Albino Guerreiro
Degree in Biochemistry

Exploring the Anti-diabetic Effect of White Wine

Dissertation to obtain the Master Degree in
Biochemistry for Health

Supervisor: Ana Teresa Serra, PhD, iBET/ITQB-UNL

Jury:
President: Doctor Pedro Manuel M. Henriques Matias
Opponent: Doctor Maria Eduardo C. Morgado Figueira
Members of the jury: Doctor Ana Teresa de Carvalho Negrão Serra
Doctor Margarida Archer Baltazar Pereira da Silva Franco Frazão

Instituto de Tecnologia Química e Biológica

November, 2016

Copyright

Acknowledgments

Gostaria de agradecer a todas as pessoas que de uma forma directa ou indirecta me apoiaram durante o desenvolvimento deste trabalho.

Primeiramente, gostaria de agradecer à Doutora Teresa Serra por me ter orientado e apoiado durante este último ano. Agradeço a paciência e a motivação durante as fases mais difíceis, os conselhos e por me ter ajudado a crescer profissionalmente e pessoalmente.

Agradeço também à Doutora Catarina Duarte pela oportunidade de desenvolver o meu trabalho no Laboratório de Nutracêuticos e Libertação Controlada do iBET.

Agradeço a todos os membros do grupo de Nutracêuticos e Libertação Controlada, em especial à Inês, Joana, Maria João e Ana Nunes por tudo o que me ensinaram e ajudaram ao longo das diferentes partes do trabalho.

A todas as minhas colegas futuras mestres. À Lucília pela motivação e pela companhia nas longas viagens. Especialmente à Ana pelos conselhos, ajuda e apoio incondicional. À Carolina por ser a "Coleguinha do Laboratório", pela entreajuda, o incentivo, o companheirismo e a cumplicidade desde o primeiro dia, que facilitaram de alguma forma a execução deste trabalho.

A todos os meus amigos e colegas de mestrado do ITQB, em especial à Joana Gomes e à Diana Sousa pelo grande apoio e motivação. Agradeço também aos meus amigos mais próximos que me apoiaram, em especial à Sara Gomes e Patrícia Marques.

Às minhas colegas de casa, Filipa e Inês, que de uma forma mais indirecta contribuíram para que este ano se tornasse mais fácil. Obrigada pelo apoio.

Ao Zé Pedro pelo apoio incondicional, paciência, ajuda, por estar sempre ao meu lado e me fazer sempre sorrir.

Aos meus pais por me terem deixado escolher este caminho, pela paciência inesgotável e por me terem apoiado sempre ao longo deste percurso. À minha tia pelo incentivo e motivação, que me ajudou a ultrapassar muitos obstáculos. À minha avó.

Abstract

Several clinical studies have shown that moderate alcohol consumption is inversely associated with the risk of developing Type 2 Diabetes due to the positive action of alcohol on specific biomarkers of the disease. Wines are very promising anti-diabetic beverages not only for their alcohol content but also for their composition in phenolic compounds. In this area, red wines have been extensively studied existing reports on their anti-diabetic potential. However, there are no studies regarding white wine, even though it is known to have similar or higher concentrations of hydroxycinnamic acids, which are potential anti-hyperglycemic agents.

The aim of this thesis was to investigate the anti-hyperglycemic potential of white wines using chemical and cell-based assays. For this purpose, four wines were characterized in terms of phenolic composition and their capacity in inhibiting carbohydrate-digestion enzymes and glucose transporters using a Caco-2 cell model.

Results showed that phytochemical composition varied greatly among all wines. Aveleda Alvarinho presented a higher concentration and more variety of phenolic compounds. This wine also showed the ability to inhibit α-amylase (IC_{50} 199.5 mg GAE/L) and α-glucosidase, and improved the activity of acarbose when combined with this drug. Gallic acid was identified as being a major contributor for the α-glucosidase inhibition, with an IC_{50} value of 32.28 mg/L. In particular, when combined with acarbose, a synergistic effect was also observed. Additionally, the results obtained in Caco-2 cells revealed that Aveleda Alvarinho was able to inhibit glucose transporters, namely SGLT1 and GLUT2, in a dose dependent-manner (IC_{50} 79.5 mg GAE/L and 126.7 mg GAE/L, respectively). The digested fraction of this wine showed a slightly higher inhibition of glucose transport, whose effect was related with the phenolic metabolites produced during *in vitro* gastrointestinal digestion.

Results of this thesis demonstrated that white wine can be considered as a promising source of new natural anti-hyperglycemic agents.

Keywords: Type 2 diabetes; White wine; Polyphenols; SGLT1 and GLUT2 transporters; Carbohydrate-digestion enzymes; Caco-2 cells

Resumo

Estudos clínicos mostraram que o consumo moderado de álcool se relaciona inversamente com o risco de desenvolver diabetes tipo 2, devido à acção benéfica do álcool em biomarcadores da doença. Os vinhos são bebidas anti-diabéticas muito promissoras, não só pelo seu conteúdo alcoólico, mas também pela sua composição em compostos fenólicos. A investigação nesta área tem incidido no vinho tinto, existindo vários estudos sobre o seu potencial anti-diabético. Contudo não existem estudos em vinho branco, mesmo tendo sido reportado concentrações semelhantes ou maiores de ácidos hidroxicinâmicos, reconhecidos pelo seu potencial anti-hiperglicémico.

O objectivo desta tese foi o de investigar o potencial anti-hiperglicémico dos vinhos brancos através de ensaios químicos e celulares. Deste modo, quatro vinhos foram caracterizados quanto à sua composição fenólica e à sua capacidade em inibir enzimas da digestão de hidratos de carbono e transportadores de glucose usando células Caco-2.

Os resultados demonstraram que a composição fitoquímica variou grandemente entre os vinhos testados. O Aveleda Alvarinho apresentou uma concentração mais elevada e uma maior variedade em compostos fenólicos. Este vinho também demonstrou capacidade em inibir α-amilase (IC_{50} 199,5 mg EAG/L) e α-glucosidase e, em combinação com acarbose, aumentou a actividade desta. Foi demonstrada a contribuição do ácido gálico na inibição da α-glucosidase, com IC_{50} de 32,28 mg/L e, quando combinado com a acarbose, foi observado um efeito de sinergia. Resultados obtidos em células Caco-2 demonstraram a capacidade do Aveleda Alvarinho em inibir transportadores de glucose, SGLT1 e GLUT2 (IC_{50} 79,5 mg EAG/L e 126,7 mg EAG/L, respectivamente), sendo o efeito dependente da dose. A fração digerida do vinho demonstrou uma inibição ligeiramente superior dos transportadores de glucose, cujos efeitos se relacionam com os metabolitos fenólicos produzidos durante a digestão *in vitro*.

Os resultados desta tese demonstraram que o vinho branco pode ser considerado uma fonte promissora de agentes naturais anti-hiperglicémicos.

Palavras-Chave: Diabetes tipo 2; Vinho branco; Polifenóis; Transportadores SGLT1 e GLUT2; Enzimas da digestão de hidratos de carbono; Células Caco-2

Table of Contents

Figure Index

Table Index

Abbreviations

CAA	Cellular Antioxidant Activity
CF	Concentration Factor
CI	Combination Index
DAD	Diode Array Detector
FBS	Fetal Bovine Serum
GAE	Gallic Acid Equivalent
GLUT2	Glucose Transporter 2
HORAC	Hydroxyl Radical Adverting Capacity
HPLC	High Performance Liquid Chromatography
IC_{50}	Half Maximal Inhibitory Concentration
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate-Buffered Saline
PKC	Protein Kinase C
p-NPG	4-Nitrophenyl α-D-glucopyranoside
RPMI	Roswell Park Memorial Institute Medium
SD	Standard Deviation
SGLT1	Sodium-Dependent Glucose Cotransporter 1
SPE	Solid Phase Extraction
TPC	Total Phenolic Content

1. Introduction

1.1. Diabetes

In the past decades, diabetes mellitus has become a worldwide health emergency. Data from the World Health Organization (WHO) shows that high blood glucose is the third leading risk factor for premature death globally, after high blood pressure and tobacco, and behind physical inactivity and obesity [1]. Nowadays, 415 million adults are estimated to suffer from this disease and 318 million are at risk of developing diabetes from impaired glucose tolerance. Last year, 5 million adults have died from diabetes and these alarming numbers are predicted to increase to 642 million adults in 2040 (Figure 1.1) [2].

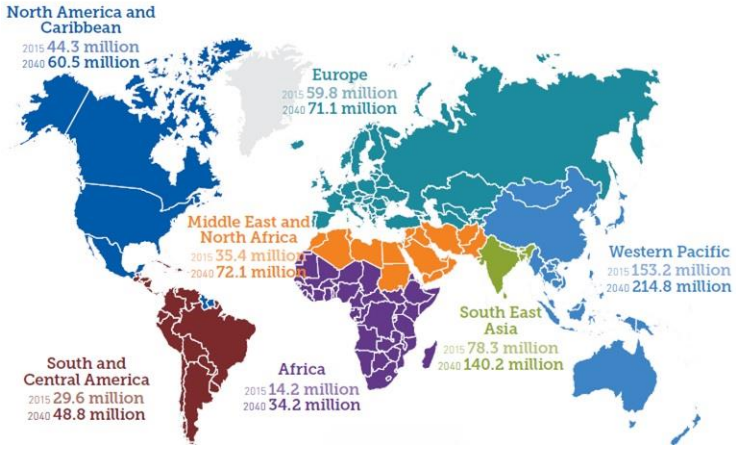

Figure 1.1: Worldwide diabetes prevalence (data from 2015) and projections for 2040. Adapted from IDF Diabetes Atlas, 7thedition [2].

Portugal is no exception and more than 1.3 million people with age between 20 – 79 years old suffer from this condition, and only 56% is properly diagnosed [3]. Diabetes was responsible for 4% of the deaths in 2014, being the 5th cause of death in the country [3,4].

Diabetes is a group of metabolic disorders characterized by chronic hyperglycemia (high blood glucose levels) which causes long-term damage, dysfunction and failure of various organs. Deficient insulin action is the basis of all anomalies in carbohydrate, lipid and protein metabolism, and it can result from a deficient insulin secretion or from a reduced insulin response of the tissues. Frequently, both problems coexist in the same patient and it is not possible to determine which caused the hyperglycemia [5].

The disease mostly manifests itself in one of two categories: type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM), but there are other types such as gestational diabetes or other specific genetic defects that cause a form of diabetes. T1DM is an autoimmune disease that affects 5 – 10% of all diabetic cases. This type of diabetes is characterized by a cellular-mediated autoimmune destruction of the pancreatic β-cells, leading to an insulin deficiency with little or no secretion. Patients with T1DM, suffer or will suffer from ketoacidosis and are dependent of insulin intake.

On the other hand, T2DM accounts for 90 – 95% of the cases and it is characterized by an insulin resistance. In these patients, autoimmune destruction of β-cells does not occur, and they usually

do not need insulin intake to survive. Because the hyperglycemia develops gradually, patients do not present the classic symptoms of diabetes at the first stages of the disease and do not get properly diagnosed early [5]. Type 2 diabetes is the result of genetic predisposition and environmental factors. In fact, epidemiological studies evidence that the worldwide diabetes epidemic faced by the 21th century society is due to lifestyle [6]. The lifestyle of developing societies has increased the prevalence of overweight and obesity, which resulted in the arising of the metabolic syndrome, a combination of pathological conditions including obesity, hypertension, dyslipidemia, insulin resistance, glucose intolerance and hyperglycemia (impaired glucose tolerance). The metabolic syndrome is related with an augmented risk of diabetes, as well as cardiovascular diseases, and several studies show that the syndrome usually predicts future diabetes [7,8]. There is no cure for diabetes, but the resulting complications can be ameliorated through diet and lifestyle modifications [6].

Nowadays, there is an increasing interest in the role of post-prandial hyperglycemia in T2DM. Repeated high post-prandial hyperglycemia is linked to several micro and macrovascular complications, being strongly associated with the risk of cardiovascular disease [9]. Over the years, multiple studies demonstrated that post-prandial hyperglycemia is linked to a severe progression of cardiovascular mortality risk and its treatment reduces the incidence of new cardiovascular events [10–12], as well as decreases the progression of several microvascular complications (such as renal failure and blindness due to retinopathy) . In consequence, prevention and management of post-prandial hyperglycemia has been a major issue in diabetic treatment. There are several therapeutic approaches available to control post-prandial hyperglycemia. Inhibitors of glycoside hydrolases (GH) such as α-glucosidase and α-amylase are already recognized approaches [13], as well new targets like intestinal glucose transporters, SGLT1 and GLUT2 [14,15].

1.2. Carbohydrate Digestion and Absorption in the Human Small Intestine

Complex dietary carbohydrates cannot be directly absorbed by enterocytes in the small intestine epithelium. First of all, dietary carbohydrates have to be fully degraded by glycohydrolases. Salivary and pancreatic α-amylase cleave the complex carbohydrates, starch, in small oligosaccharides that are further digested by brush border α-glucosidases into monosaccharides. This cascade of catalytic reactions in the intestine occurs concomitantly [16]. Monosaccharides are then transported across the intestinal epithelium via specific transporters (SGLT1 and GLUT2 transport glucose, and GLUT5 transports fructose) into the blood stream. It is in the first segments of the human intestine that there is a higher affinity for glucose molecules, and consequently a higher velocity of transport that ceases in the colon. The newly absorbed monosaccharides are then distributed to various organs and tissues, and take part in numerous metabolic pathways [17].

1.2.1. Carbohydrate-Degrading Enzymes: α-Amylase and α-Glucosidase

α-Amylases (EC 3.2.1.1) are GH 13 family enzymes, that catalyze the endohydrolysis of the α-(1,4)-glycosidic bonds of starch. Starch, that constitutes 40 – 50% of the ingested carbohydrates in the Western diet, is a glucose polymer that consists of two different molecules: amylose, a linear chain of α-(1,4)-glucose linked units, and amylopectin, a branched molecule with a backbone of α-(1,4)- linked glucose and branches of α-(1,6)-glucose units. In mammalians, there are two isoforms of α-amylase: salivary, secreted by the parotid gland, and pancreatic, secreted by the pancreas into the small intestine [18]. Salivary α-amylase only has activity during food mastication in the mouth, because the low stomach pH promptly inactivates the enzyme [16]. On the other hand, pancreatic α-amylase is the first step of the carbohydrate digestion in the small intestine. It is a glycoprotein of 57 kDa that consists of 3 domains: A, B and C, and has 3 functionally important sites: the active site, the calcium-biding site and the chloride-biding site. These functionally important sites are constituted by structural elements of domains A and B. Domain C appears to be an independent domain, with unknown function. The enzyme active site is located in the cleft between domains A and B and contains 5 subsites, with the catalytic site positioned at subsite 3. Glucose residues can bind to subsites 1 or 2, and cleavage occurs between the first and second or the second and third glucose residues. α-Amylase requires one calcium ion to stabilize the active site cleft, and chloride ions to maintain activity [18,19].

The products of starch digestion by α-amylase are dextrins (maltose, maltotriose and branched oligosaccharides) and their further breakdown into monosaccharides will be accomplished by the intestinal brush border enzymes α-glucosidases [18].

α-Glucosidases (EC 3.2.1.20) are a group of hydrolases from family GH 31 that catalyze the exohydrolysis of terminal, non-reducing α-(1,4)-glycosidic bonds of polysaccharides, releasing α-D-glucose. These enzymes are commonly constituted by four major domains: an N-terminal domain, a $(\beta/\alpha)_8$-barrel catalytic domain and two C-terminal domains [20].

In the small intestine there are four membrane bound glycohydrolases: trehalose, maltase-glucoamylase, lactase and sucrase-isomaltase. Dextrins suffer further degradation by combined activity of maltase-glucoamylase and sucrase-isomaltase complexes, each containing two active sites. In humans, sucrase-isomaltase is more abundant than maltase-glucoamylase, accounting for about 10% of all brush border proteins, and therefore responsible for 80% of the maltase activity. Both subunits have maltase activity and this is also the only enzyme in the small intestine to have sucrase activity, essential to sucrose digestion. The catalytic mechanism and active site

architecture of most α-glucosidases occurs by very similar reaction mechanisms [16]. Briefly, the hydrolysis reaction of the glycosidic linkage occurs by cleavage of the bond between the anomeric carbon of the glycosyl residue and the glycosidic oxygen (Figure 1.2) [21].

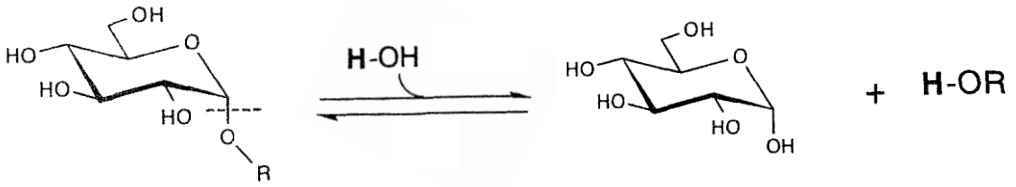

Figure 1.2: Scheme of α-glucosidase hydrolytic reaction of an α-(1,4)-glycosidic bond. R is a reducing sugar residue. Adapted from Chiba, 1997 [21].

Since the activity of α-glucosidases is the final step before glucose absorption on the small intestine epithelium occurs, their inhibition is an effective way of decreasing post-prandial hyperglycemia peaks [22,23]. Acarbose, a pseudo-tetrasaccharide, is the first commercialized α-glucosidase inhibitor and a very important anti-diabetic oral drug. The drug acts through a competitive and reversible inhibition of the brush border α-glucosidases and pancreatic α-amylase, delaying the carbohydrate digestion, which reduces the rate of glucose absorption [24]. However, the use of α-glucosidase inhibitors as acarbose causes serious side-effects such as abdominal distention, meteorism, flatulence and diarrhea [24–26]. It has been proposed that this side effects are due to a strong inhibition of the complex carbohydrates digestion, more precisely of an excessive pancreatic α-amylase inhibition, which allows the undigested carbohydrates to reach the colon, where abnormal fermentation results in intestinal gas, and consequently the side effects referred above [24,27,28]. Thus, it is necessary to search for alternative ways that can display the same α-glucosidase inhibitory activity without such side effects. Compounds from natural sources that display this anti-hyperglycemic bioactivity have been studied, yielding promising results [29].

1.2.2. Glucose Transport: SGLT1 and GLUT2

Hydrophilic molecules, like sugars, cannot permeate freely across the lipid bilayer of the plasma membrane in eukaryotic cells. Transmembrane proteins from two different types are responsible for glucose transport in the organism: the sodium-coupled glucose cotransporters (SGLT) and the facilitated glucose transporters (GLUT). Both transporters belong to the sodium carrier gene superfamily (*SLC*), SGLT being transporters part of *SLC5* gene family and GLUT transporters part of *SLC2* family [30]. SGLT1 is a high-affinity and low-capacity transporter, responsible for glucose entrance in the enterocytes, while GLUT2 is a low-affinity but high-capacity transporter responsible for the transport of glucose and fructose into the blood and also into the enterocyte, at high luminal sugar concentrations [31].

SGLT1 is an integral membrane protein, constitutively expressed in the brush border enterocytes, responsible for the active transport of glucose. It can also transport galactose but with much lower affinity [30]. The *SLC5* gene codes for proteins of 75 kDa, approximately. The structure of the transporter is not fully disclosed but it is already known that is a polytopic membrane protein, with two glucose binding sites on the C-termini, one on the extracellular face and another on the intracellular face of the protein. It is composed of 14 transmembrane helices, with the N- and C-

termini facing the extracellular side of the plasma membrane. Glucose binds to the protein through hydrogen bonds, between the polar residues of the sugar and the polar side-chains of the binding site residue. The mechanism of transport is a symport, where the protein couples the transport of two sodium ions and one glucose molecule, against the glucose gradient, with an energy cost (Figure 1.3). Across the brush border membrane, a sodium electrochemical potential gradient is established, maintaining the driving force for glucose transport. It is the entrance of sodium ions with the glucose molecules into the cells and its transport into the blood by the Na/K-pump that maintains the gradient, providing the necessary energy [32].

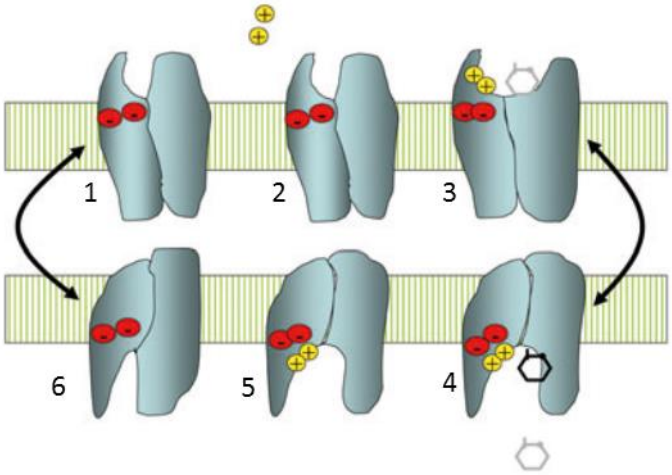

Figure 1.3: Model of Na/glucose cotransport by SGLT1. SGLT1 is negatively charged with a valence of -2, depicted in red. First of all, two sodium ions (yellow) bind to the transporter (1-2), producing a conformational change that allows the biding of one glucose molecule (2-3). Both the sodium ions and glucose are transported across the membrane (3-4) and, due to the low intracellular concentration of sodium, the sodium ions and glucose dissociate from the protein, entering the cell (4-5). Then, the protein binding sites re-orientate from the inner to the external membrane surface (6-1). Taken from Wright *et al.*, 2007 [32].

There has been an increasing interest in developing new approaches to regulate post-prandial glucose peaks by inhibiting the glucose absorption in the intestine through SGLT1 [17]. Nakazawa, in 1922, was the first to demonstrate that glucose transport could be reduced using a phenolic compound named phloridzin, a flavonoid glycoside mostly found in apples [33]. Decades later, in 1967, Alvarado demonstrated that the glucose transport reduction showed by Nakazawa was due to a potent competitive inhibition of SGLT1 in the intestinal brush border [34]. In recent years, phloridzin-like SGLT1 inhibitors have been developed by the pharmaceutical industry, and some are undergoing preclinical and clinical trials, however severe side effects due to the potent inhibition of the transporter can occur, such as diarrhea and other gastrointestinal problems [32].

The two components of glucose absorption in the small intestine, one active and another “diffuse”, were established by Donhoffer in 1932 [35]. The importance of the “diffuse” component was showed by Madara & Pappenheimer in 1987, where the authors demonstrated that it accounts for approximately 75% of the glucose absorption but there was no consensus about its origin [36,37]. In the classical model of glucose absorption, GLUT2 was exclusively located at the basolateral membrane of the enterocytes and all glucose transport was managed by SGLT1. However, in recent years, it was discovered that GLUT2 could traffic to and from the apical membrane and was able to transport both fructose and glucose [31].

Structurally, the GLUT proteins are composed of twelve hydrophobic transmembrane α-helices, with de C- and N-termini facing the cell cytoplasm [30]. They perform passive transport of glucose, facilitating the equilibrium of glucose and other sugars across the membranes. In the small

intestine epithelium, GLUT2 is constitutively expressed in the basolateral membrane of the enterocytes and it is responsible for the passive transport of glucose into the blood stream, although a fraction of the intracellular glucose seems to be transported into the blood by endosomes, as glucose-6-phosphate [32]. The activity of GLUT2 only depends on the glucose concentration and it is not saturated at physiologic glucose concentrations [30].

As mentioned above, GLUT2 is also a very important component of glucose transport between the intestinal lumen and the enterocyte's cytoplasm [38].

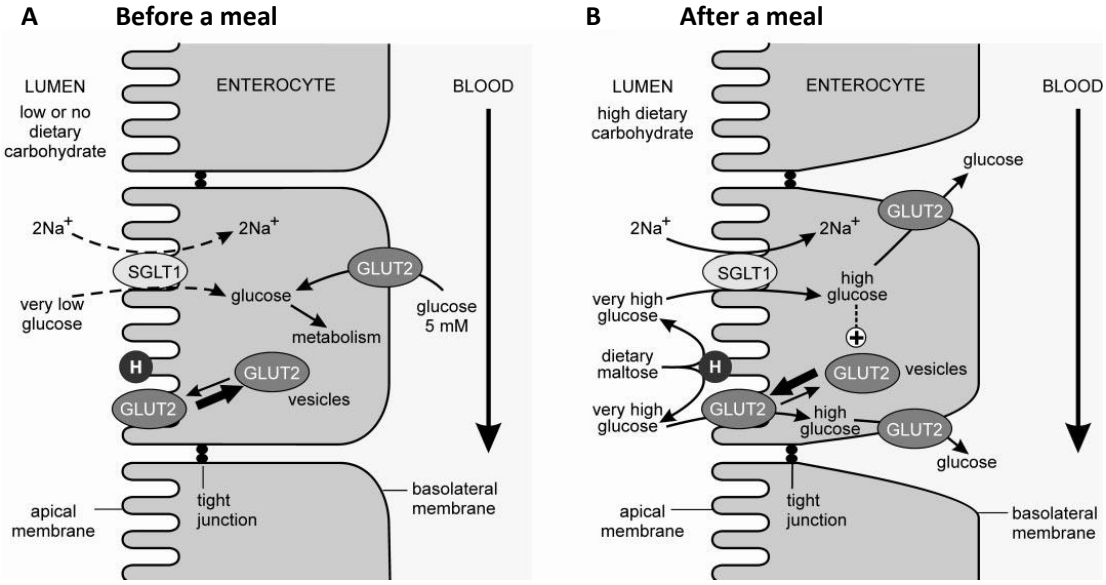

Figure 1.4: Intestinal glucose absorption model. (A) Before a meal, GLUT2 is present in the apical membrane at very low concentration and glucose transport is made only by SGLT1. Basolateral GLUT2 operates in the opposite direction, in order to supply glucose to the enterocytes so the cells can maintain their energy requirements. (B) After a meal, brush border α-glucosidases (H) hydrolyze polysaccharides and apical glucose concentration increases. Initially, glucose transport is made through SGLT1, increasing intracellular glucose concentration, which activates PKC βII. PKC βII then rapidly recruits and activate GLUT2 to the apical membrane from intracellular vesicles. At this point, GLUT2 is the major glucose absorption pathway. When the luminal glucose concentration declines, the signaling system is reversed and GLUT2 becomes inactivated and traffics away from the apical membrane. Figure taken from Kellett *et al.*, 2005 [38].

Before a meal, the sugar concentration in the intestinal lumen is very low, and the only active transporter in the brush border is SGLT1. GLUT2 is present at a very low concentration or even absent, to minimize glucose escape from the mucosa (Figure 1.4 A). After a meal, the concentration of sugars at the brush border is higher and α-glucosidases degrade oligosaccharides into glucose, augmenting the luminal glucose concentration. SGLT1 transports glucose until saturation is reached. The increasing glucose concentration at the lumen activates PKC βII which recruits GLUT2 into the apical enterocyte membrane, allowing glucose to enter the cell by facilitated transport (Figure 1.4 B). Thus, GLUT2 is responsible for the major path of absorption at high luminal glucose conditions. The transporter response to glucose is concentration-dependent and cooperative, because GLUT2 activation and protein concentration in the apical membrane increase with glucose concentration [31,38].

Apical GLUT2 recruitment is strongly linked to diet. Increased glucose transport by apical GLUT2 might be a factor in repeated insulin spikes, which contributes to insulin resistance, obesity and type 2 diabetes. Thus, apical GLUT2 is a potent therapeutic target in type 2 diabetes patients. Modulation of glucose absorption through dietary manipulation can be an important approach to reduce post-prandial glucose peaks [38].

1.3. Natural Compounds and Type 2 Diabetes

Plants were the basis of sophisticated traditional medicine, used for the treatment of a wide spectrum of diseases, with the earliest records dating from 2600 BCE, documenting the uses of 1000 plant-derived substances in Mesopotamia. Throughout the years, natural compounds have been the source of most active principles and very important lead compounds in the discovery and development of new drugs. These compounds are of very high interest for drug development not only because of their structural diversity, but also because often they possess selective and specific biological activities based on mechanisms of action. It is proposed that the bioactivity of natural products arises from the fact that all natural compounds have receptor-binding activity *in vivo*, although finding the receptor to which a given compound binds is a challenge. Even though the interest in the investigation of bioactive compounds from plants, only 6% of the higher plants have been pharmacologically studied and 15% evaluated regarding their phytochemical composition [39,40]. Nowadays, screening of natural compounds by pharmaceutical companies has decreased because of the difficulties of access and supply, the complexities of the chemistry of natural products and the slowness of working with this products, which is counter-productive since these compounds were the source for a considerable number of important drugs that are presently on the market [39,40].

Another approach that many pharmaceutical and biotechnological companies have to take advantage of these natural bioactive compounds is the development of nutraceuticals and functional foods. The term nutraceutical was first defined by Stephen DeFelice in 1979 as a “food, or parts of food, that provide medical or health benefits, including the prevention and treatment of disease” [42]. A nutraceutical is a diet supplement that contains a concentrated form of a bioactive compound, presented in a non-food matrix, in dosages that exceed those that could be obtained from normal food, while a functional food is a food that when regularly consumed exerts a specific beneficial effect beyond its nutritional properties, the effect of which has to be scientifically proven [43].

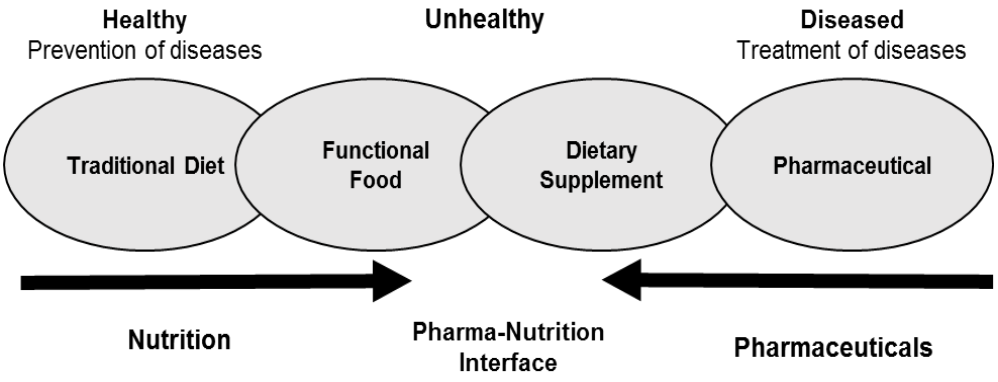

Figure 1.5: Interface between pharmaceuticals and nutrition. Adapted from Eussen, S. *et al.*, 2011 [44].

Functional foods and nutraceuticals make the interface between pharmaceuticals, that are traditionally utilized to treat or alleviate symptoms of diseases, and nutrition, of which the first aim is to provide the body with an optimal balance of nutrients needed for good health, thus preventing diseases (Figure 1.5). Since today’s chronic diseases have a multifactorial origin, a treatment approach complementing pharmaceuticals and nutrition is the most successful [44].

These health-promoting products, such as nutraceuticals and functional foods, usually contain bioactive compounds that are constituents of plant-derived foods that present health-promoting

effects. Part of the capacity that some plant-derived foods have to reduce the risk of chronic diseases has been associated to phytochemicals. Phytochemicals are non-nutrient secondary metabolites that exist in fruits and vegetables and have a low potency when compared to pharmaceutical compounds, but when ingested regularly have a noticeable long-term physiological effect. These bioactive constituents can be incorporated into the products as food extracts or phytochemical enriched extracts, to which a given beneficial physiological function has been attributed. The most common phytochemicals found in these products are polyphenols such as anthocyanins, flavonols, stilbenes and hydroxycinnamic acids, but glucosinolates and terpenoids are found often as well [43].

Dietary polyphenols can be found in vegetables (broccoli, onion, cabbage), fruits (grapes, apples, cherries and a wide variety of berries), legumes (soybeans), cereals, plant derived beverages (tea, coffee, wine) and chocolate [45]. There are over 8000 plant phenolic compounds identified and they have diverse and important physiological and morphological functions, such as phytoalexins, antifeedants, pigmentation, protection against UV light and antioxidants [46]. Phenolic compounds are characterized by having one (monophenolic) or more than one aromatic rings (polyphenolic), bearing at least one hydroxyl group. They can have from a simple structure, a simple phenolic acid or alcohol, to a complex high molecular mass polymer, and usually are conjugated with sugars and organic acids [46,47].

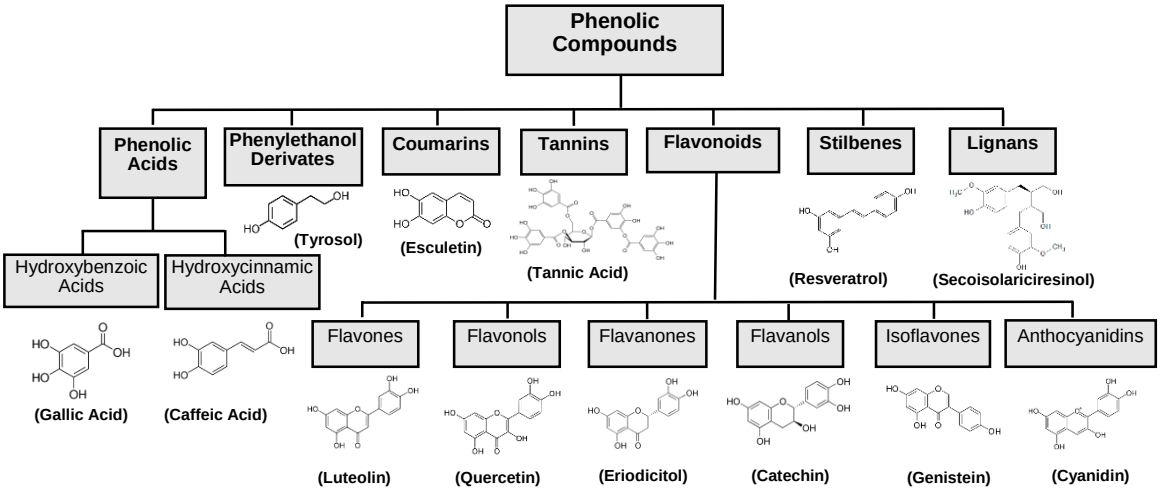

Figure 1.6: Classification and chemical structures of phenolic compounds reported in the literature as having health benefits. Adapted from Kim *et al.*, 2016 [48].

According to the number of phenol rings and the structural elements connecting the rings, polyphenols can be divided into classes: flavonoids, phenolic acids, phenylethanol derivates, tannins, stilbenes, lignans and coumarins (Figure 1.6). The diversity of structures and the different characteristics of the phenolic compounds originates the different recognized bioactivities [45–47].

In recent years, it has been observed that phytochemical extracts or plant-derived foods produce a different effect than the pure isolated bioactive compounds [49]. Polyphenols can interact additively, synergistically or antagonistically with other phenols and other food components, as well as with drugs, both from natural or synthetic origin [50]. Several studies on different medical conditions have demonstrated the increased efficacy of the co-administration of pharmaceutical drugs combined with polyphenols, as well as different combinations of polyphenols, and their value as therapeutic alternatives. These therapeutic combinations present diminished side effects and lower toxicity than increased drug concentrations [51–56].

The consumption of a diet abundant in plant-derived products, rich in polyphenols, has been inversely correlated with the incidence and prevalence of type 2 diabetes. Epidemiological and clinical studies have demonstrated that T2DM can be prevented and the symptoms ameliorated by lifestyle modifications such as increased physical activity, weight loss and a balanced diet rich in fruits and vegetables. This fact has raised interest in discovering which type of polyphenols are associated with the beneficial effects and which are their mechanisms of action [48,57,58]. Summarized in table 1.1 are several phenolic compounds that have been shown to have an anti-diabetic role, mostly by producing an anti-hyperglycemic effect, and the underlying mechanism of action.

Table 1.1: Reported anti-hyperglycemic effect of different types of phenolic compounds. ↑ increase; ↓ decrease.

	Phenolic Compound	**Anti-Diabetic Effect**	***In vivo***	***In vitro***	**Reference**
Flavonols	Catechin	↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (SGLT1)	✓	✓	[59–61]
	Epicatechin	↓ Intestinal glucose absorption (SGLT1), Protection of β cells in isles or pancreas, ↑ Insulin secretion	✓	✓	[61–63]
	Quercetin	↓ α-Amylase activity, ↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (GLUT2 and SGLT1)	✓	✓	[61,64–66]
	Cyanidin	↓ α-Amylase activity		✓	[64]
Phenolic Acids	Caffeic Acid	↓ α-Amylase activity, ↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (SGLT1)	✓	✓	[59,60,67–69]
	Chlorogenic Acid	↓ α-Amylase activity, ↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (SGLT1 and GLUT2)	✓	✓	[59,60,67,70–73]
	p-Coumaric Acid	↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (SGLT1 and GLUT2)		✓	[68,73]
	Ferulic Acid	↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (SGLT1), ↑ insulin secretion, Induction of hepatic glucokinase activity	✓	✓	[60,68,69,72,74,75]
	Gallic Acid	↓ α-Glucosidase activity		✓	[68]
Tannins	Tannic Acid	↓ α-Amylase activity, ↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (SGLT1)		✓	[60,68,70]
Stilbenes	Resveratrol	↓ α-Glucosidase activity, ↓ Intestinal glucose absorption (SGLT1)		✓	[76,77]
	ε-Viniferin	↓ Intestinal glucose absorption (SGLT1)		✓	[77]

1.4. White Wine and Health

White wine is one of the 100 richest dietary sources of polyphenols, which have been linked to produce improvement on health [78,79]. For many years, the so called “French Paradox” has been studied: despite a high consumption of saturated fats, the mortality in France from cardiovascular events is low, in comparison with other countries with the same eating habits. Various epidemiological studies have proposed that the regular and moderate consumption of wine is responsible for these beneficial effects on health, not only in cardiovascular diseases, but also in lowering the declining of cognitive function in neurological diseases, lowering the risk of developing diabetes mellitus and cardiometabolic complications in diabetic patients (Figure 1.7) [78,79,80]. The biological effect of wine is mostly due to its wide variety of phenolic compounds, therefore having a wide range of potential biological targets [79].

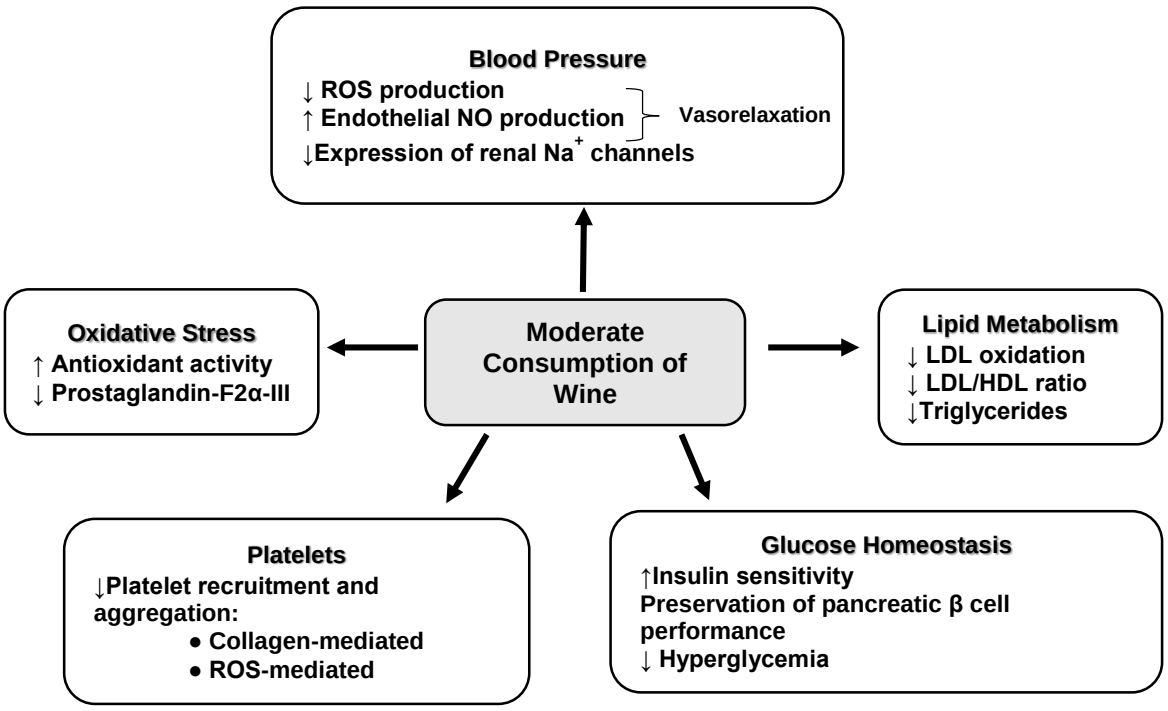

Figure 1.7: Biological effects produced by the consumption of wine described in the literature. Abbreviations: ROS, reactive oxygen species; NO, nitric oxide; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol. Adapted from Artero, *et al.*, 2015 [79].

1.4.1. Phytochemical Composition of White Wine

White wine is a mixture of different components: water, ethanol, glycerol, polysaccharides, organic acids and phenolic compounds [79]. Non-volatile phenolic compounds are intrinsic components of grapes and their derivatives, such as wine. The different phenolic composition between different wines is due to the fact that phenolic composition is very dependent on the grape variety and maturity, environmental factors, as well fermenting and aging conditions [82].

Wine phenolics are usually divided into two categories: flavonoids and non-flavonoids. Flavonoids are the major polyphenolic class in red wines and exist in white wines in a considerably lower concentration. This happens because red wine production involves maceration and flavonoids are extracted during the process from skin and other grape components, while in white wine production the maceration step is avoided. These phenolic compounds mostly occur in wine as flavonols, flavones and anthocyanidins [79,83].

Non-flavonoid phenolic compounds are mostly found as hydroxycinnamic acids, hydroxybenzoic acids, stilbenes and hydrolysable tannins. Hydroxycinnamic acids are the major class of phenolic compounds in white wine. In grapes they are the third most abundant class of phenolics, existing as esters of tartaric acid - caftaric, coutaric and fertaric acids. As wine is an aqueous acidic solution, the esters are hydrolyzed releasing the simple acids – caffeic, *p*-coumaric and ferulic acids- existing there in both forms. Hydroxybenzoic acids are not as common in wine as hydroxycinnamic acids but several have been already identified. Gallic acid is fairly abundant as it is the precursor of all the hydrolysable tannins. Hydrolysable tannins and stilbenes are another minor classes of phenolics in white wine. Hydrolysable tannins are complex polyphenols that can be found as gallotannins and ellagitannins, which come from aging in oak barrels. Resveratrol is the most mentioned and abundant stilbene present in wine, forming oligomeric forms known as viniferins [82–84]. Throughout the years, the health improvement effects of wine have been attributed to the presence of resveratrol, mostly regarding its presence in red wine and its beneficial effects in cardiovascular diseases [79,85,86]. However, studies have brought evidence that the beneficial effects of wine are in reality due to other phenolic acids and not only resveratrol [87].

1.4.2. White Wine and Diabetes

Several of phenolic compounds that exist in white wine have already been reported to present a promising effect in type 2 diabetes by diminishing the post-prandial glucose peak, producing an anti-hyperglycemic activity. In fact, epidemiological and clinical studies have reported that white wine can lower glucose plasma concentration and lower the risk of developing type 2 diabetes, while not presenting any adverse effects in diabetes type 2 patients. The studies are summarized in table 1.2.

Table 1.2: Summary of cohort studies related with white wine consumption and the reduced risk of type 2 diabetes. ND, not defined

Subjects	Study/Method	Dose/Duration	Effects/Results	Reference
18 patients with T2DM	Randomized controlled trial	**Dosage:** 240 mL **Duration:** 30 days	White wine moderate consumption did not present any harmful effect on patients and lowered fasting serum insulin.	Bantle, *et al.*, 2007 [88]
224 patients with T2DM	Randomized controlled trial	**Dosage:** 150 mL **Duration:** 2 years	Observed benefit from both red and white wine in glycemic control. No adverse effects observed from wine intake.	Gepner, *et al.*, 2015 [89]
36 527 adults	Observational study. Dietary assessment by questionnaire	**Dosage:** ND **Duration:** 4 years	Incident cases of T2DM were identified. Wine, both red and white, consumption was associated with a lower risk of developing T2DM in women.	Hodge, *et al.*, 2006 [90]
20 healthy males	Randomized controlled trial	**Dosage:** 400 mL **Duration:** 2 weeks	Consumption of red and white wine resulted in a reduction in plasma glucose concentration.	Lavy, *et al.*, 1994 [91]

In recent years, studies with white wine pomace and other derivatives have brought evidence that phenolic compounds from white grapes can modulate the carbohydrate digestion by α-amylase and α-glucosidase inhibition [92,93]. There is preliminary studies showing that white wine compounds are able to inhibit these enzymes, however these assays are mainly carried out with α-glucosidase and α-amylase derived from yeast [94]. With respect to glucose transport across the small intestine epithelium, there are studies including phenolic compounds that are known to exist in white wine, demonstrating their potential in modulating glucose absorption [61,65,77]. However, there are no studies assessing the effect of white wine in intestinal glucose absorption in this context.

White wine is a promising study model for the modulation of carbohydrate digestion and absorption in the small intestine, which can have a beneficial effect in type 2 diabetes patients.

1.5. Aim of the Thesis

Wine contains phenolic compounds recognized for their antioxidant and anti-hyperglycemic proprieties. Regarding diabetes, particular attention has been given to red wine due to its high polyphenolic concentration. On the contrary, few studies have been done with white wine. However, given its rich phytochemical composition in phenolic acids with recognized anti-hyperglycemic activity, white wine is a promising candidate for study.

The aim of this thesis is to explore the anti-hyperglycemic potential of white wines.

In order to achieve the proposed goals, the work was divided in four tasks, giving rise to two parts, and it is schematically represented in Figure 1.8:

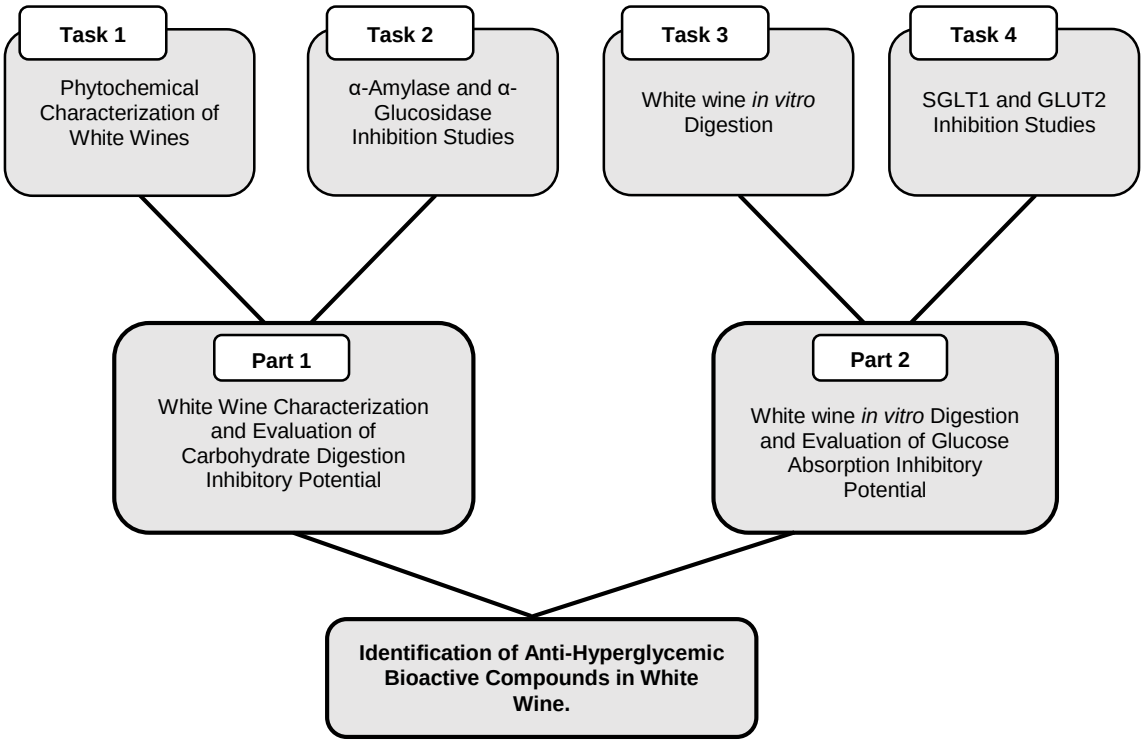

Figure 1.8: Structure of the thesis.

In the first part of the thesis the phytochemical characterization of the four white wine samples was carried out. The enzymatic assays were also performed in this part: α-amylase inhibition dose-response curves were done in order to determine the IC_{50} of all samples and α-glucosidase inhibition assessment was also done for the four samples. Synergy studies between wine and acarbose were pursued, as well as identification of the possible bioactive compounds responsible for the observed effect and its synergy with acarbose. The results obtained in the first part of the thesis allowed the selection of the most promising white wine to pursue the remaining work. The second part focused on the SGLT1 and GLUT2 inhibition using Caco-2 cells as a model for small intestinal epithelium, with both *in vitro* digested and dealcoholized white wine. Dose-response curves were performed for both samples and IC_{50} values determined, as well as screening for identification of bioactive compounds that display glucose absorption inhibitory potential.

This work follows on the project "White Wine and Health" funded by Aveleda, where the phytochemical composition of white wines was evaluated, as well its antioxidant potential. Preliminary results on enzymatic studies prompted interest in exploring the anti-hyperglycemic potentialities of white wine.

2. Experimental Procedure

2.1. Wine Samples

Five different white wines were used in this work, four from Aveleda (Douro) and one from Portalegre Wine Cooperative (Alentejo), different in its origin and in the grape varieties utilized in its manufacture. The wines tested from Aveleda were Quinta da Aveleda (2011, Loureiro and Alvarinho grape varieties, 11% alcohol volume), Aveleda Douro Doc (2011, Gouveio, Malvasia and Moscatel grape varieties, 12% alcohol volume) and Aveleda Alvarinho (2011 and 2014, Alvarinho grape varieties, 12% alcohol volume). From Portalegre Wine Cooperative, Conventual (2011, Arinto, Fernão Pires and Roupeiro grape varieties, 13.5% alcohol volume) was the only wine tested.

2.1.1. Phenolic Fraction

White wine has a small percentage of endogenous sugars, which can interfere with α-glucosidase assays.

Polyphenolic rich fractions, devoid of endogenous sugars, were obtained by solid phase extraction (SPE) as described by Manzano *et al*., with some modifications [73]. SPE cartridges (Supelclean™ LC-18 SPE Tubes, Supelco) were conditioned with 4 mL of white wine and washed with 10 mL of Milli-Q water, in order to remove the endogenous sugars. The polyphenolic rich fraction was eluted with 10 mL of methanol and concentrated in a rotary evaporator at 40°C under reduced pressure.

2.2. Phytochemical Characterization

2.2.1. Total Phenolic Content by the Folin-Ciocalteau Method

Quantitative determination of the polyphenolic concentration in white wines was performed according to the Folin-Ciocalteau colorimetric method [95], as previously described by Serra, *et al.* [96]. In a 96-well microplate, 3 µL of wine samples were added to 237 µL of distilled water and oxidized with 15 µL of Folin-Ciocalteau reagent. The reaction was neutralized with 45 µL of sodium carbonate (Na_2CO_3). After a 30 minutes incubation at 37 °C, the absorbance of the samples was measured in a microplate spectrophotometer (Spark® 10M, Tecan, Switzerland) at 765 nm. Gallic acid (Fluka, Germany) was used as an external standard for the calibration curve (0 – 800 mg/L), and the results were expressed as means of triplicates (mg of gallic acid equivalents per liter of wine – mg GAE/L) ± standard deviation.

2.2.2. Polyphenolic Analysis by HPLC

Qualitative and quantitative profiles of polyphenols present in the samples were determined using HPLC-DAD by the Analytical Group of iBET (Oeiras, Portugal) coordinated by Dr. Rosário Bronze. The system used was a Thermo Finnigan (Surveyor model) equipped with an autosampler, pump and photodiode-array detector (PDA). Chromatographic separation of compounds was carried out on a Lichrocart RP-18 column (250 x 4 mm, particle size 5 µm, Merck) and a Manu-cart® RP-18 pre-column in a thermostated oven at 35°C. The photodiode array detector was programmed for scanning between 192 and 798 nm at a speed of 1Hz with a bandwidth of 5 nm. The detection was monitored using three individual channels, 280, 320 and 360 nm, at a speed of 10Hz with a bandwidth of 11 nm. The injection volume applied was 20 µL. Chromatographic separation of phenolic compounds was carried out with solvent A – acetonitrile, water and formic acid (90:9.5:0.5 v/v/v) – and solvent B – water and formic acid (99.5:0.5 v/v) – under the following conditions: linear gradient starting at 94.4 – 83.3% solvent B in 15 minutes, 83.3-77.8% in 20 minutes, isocratic for 10 minutes, 77.8-66.70% solvent B in 55 minutes, 66.70 – 44.40% in 80 minutes, 44.0 – 0% in 120 minutes, isocratic for 15 minutes, 0 – 94.40 % in 140 minutes and isocratic for 20 minutes. The auto sampler's temperature was set at 12°C. The data acquisition system used was the Chromquest (version 4.0, Thermo Finnigan-Surveyor, USA).

Identification of compounds was achieved by comparison of retention times and spectra with pure standards within that range. Calibration curves were constructed with gallic acid, neochlorogenic acid, catechin, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid and resveratrol (0 – 50 ppm), as external standards, and results were expressed as milligrams per liter of white wine (mg/L).

2.2.3. Polyphenolic Analysis by HPLC-MS

Analyses by HPLC - MS were performed by the Analytical Group of iBET (Oeiras, Portugal) coordinated by Dr. Rosário Bronze. Identification and quantification of the compounds present in white wines was performed by reverse phase LC (Waters Alliance 2695 Separation Module, Waters®) followed by negative ion electrospray ionization with MS/MS (MicroMAss Quattromicro® API, Waters®).

2.3. Enzymatic Assays

2.3.1. α-Amylase Inhibition Assay

The α- amylase (EC 3.2.1.1) inhibitory capacity of white wines was assessed through the iodine-starch colorimetric assay, as described by Al-Dabbas *et al.* [97]. Briefly, 60 µL of white wine, in a range of different concentrations, were incubated with 200 µL of a starch solution (400 µg/mL) at 37°C for 5 minutes. Then, 20 µL of a 50 µg/mL solution of α-amylase from porcine pancreas (Sigma Chemical Co., USA) prepared in PBS (Sigma-Aldrich, USA) were added to the reaction mixture, as well as 20 µL of 0.01 M PBS. The mixture was incubated at 37°C for 7.5 minutes. Finally, 200 µL of 0.01 M iodine solution and 1 mL of distilled water were added. The reaction of the iodine solution with the starch produces a purple-black color, whose absorbance was recorded at 660 nm in a spectrophotometer (Genesys10uv, Thermo Spectronic, New York, USA). The negative control of the samples was performed without addition of enzyme (20 µL of 0.01 M PBS) and a solution of 0.02 mg/mL of acarbose (Glucobay® 50, acarbose 50 mg, Bayer, Germany) was used as a positive control of the reaction.

The inhibition (%) was calculated according to equation 1:

$$\text{Inhibition (\%)} = \frac{\text{Abs blank}_{660\text{ nm}} - (\text{Abs sample without enzyme}_{660\text{ nm}} - \text{Abs sample with enzyme}_{660\text{ nm}})}{\text{Abs blank}_{660\text{ nm}}} \quad \text{Equation (1)}$$

The wine concentration that reduces α-amylase activity by 50% (IC_{50}) was determined for each sample. Dose-response curves of α-amylase inhibition (%) versus wine concentration (mg GAE/L) were plotted, and the IC_{50} values of the tested wine samples were determined using non-linear regression with GraphPad Prism® (version 6.01, GraphPad Software Inc., USA).

All assays were carried out as triplicates and the results expressed as mean of % inhibition ± standard deviation.

2.3.2. α-Glucosidase Inhibition Assay

α- Glucosidase (EC 3.2.1.20) inhibition by white wines was determined according to the method described by Mai *et al.* [98] with some modifications. The enzymes were extracted from rat intestinal acetone powder (Sigma Chemical Co., USA), source of mammalian α-glucosidases. A 5 mg/mL solution of rat intestinal acetone powder was prepared in 0.1 M maleate buffer at pH 6.9, homogenized and centrifuged (Mikro 220R, Hettich, Germany) at 5600x g during 15 minutes, at 4°C. The supernatant collected was diluted to a 1:2 ratio in 0.1 M maleate buffer pH 6.9. Twenty-five microliters of sample were preincubated with 50 µL of an α-glucosidase solution for 15 minutes at 37°C in a 96-well microplate. Then, 50 µL of 2 mM 4-Nitrophenyl α-D-glucopyranoside (Sigma-Aldrich, USA) in 0.01 M PBS (pH 6.9) were added to the mixture in order to start the reaction, and the absorbance was read at 405 nm in a microplate spectrophotometer (Spark® 10M, Tecan, Switzerland). The reaction mixture was further incubated at 37°C for 30 minutes and the reaction stopped by adding 175 µL of 1 M Na_2CO_3. The of amount *p*-nitrophenol released from the *p*-NPG at 405 nm was used to determine the enzyme activity.

For each sample at each tested concentration, a negative control was performed by adding 50 µL of 0.1 M maleate buffer (pH 6.9) instead of α-glucosidase. Therefore, any interference of the sample color in the absorbance recorded at 405 nm could be eliminated.

In order to eliminate the interferences of the reaction mixture components, all values recorded after 30 minutes incubation were corrected by subtracting the absorbance values at 405 nm recorded after the addition of p-NPG.

The inhibition (%) was calculated according equation 2:

$$\text{Inhibition (\%)} = \frac{\text{Abs blank}_{405\text{ nm}} - (\text{Abs sample without enzyme}_{405\text{ nm}} - \text{Abs sample with enzyme}_{405\text{ nm}})}{\text{Abs blank}_{405\text{ nm}}} \quad \text{Equation (2)}$$

The IC_{50} of acarbose and gallic acid were determined by plotting the dose-response curve of α-glucosidase inhibition (%) versus concentration (mg/L). The IC_{50} values were determined using non-linear regression with GraphPad Prism® (version 6.01, GraphPad Software Inc., USA).

The quantitative analysis of the dose-effect relationship between the combined effects of acarbose and gallic acid on the α-glucosidase inhibition was performed using the Chou-Talalay method [99]. Synergism, addition and antagonism were quantified through the calculation of the Combination Index (CI) using CompuSyn (Version 1.0, 2004, Combo Syn Inc., USA).

All assays were carried out at least as duplicates and the results expressed as mean of % inhibition ± standard deviation. Statistical significance was established by ANOVA using GraphPad Prism® (version 6.01, GraphPad Software Inc., USA).

2.4. *In vitro* Digestion

An *in vitro* digestion procedure, as described by Minekus *et al.* [100], was used to evaluate the transformation that white wine polyphenols suffer throughout the gastrointestinal tract. The procedure simulates digestion on the upper digestive tract and is carried out through three phases: oral phase, gastric phase and small intestinal phase.

Oral Phase

In the oral phase, 5 mL of white wine were added to 4 mL of Simulated Salivary Fluid (KCl, 15.09 mM; KH_2PO_4, 1.35 mM; $NaHCO_3$, 13.68 mM; $MgCl_2(H_2O)_6$, 0.15 mM; $NH_4(CO_3)_2$, 0.06 mM; $CaCl_2(H_2O)_2$, 1.5 mM; HCl, 1.1 mM; pH 7). The pH was adjusted to 7 by adding 6 M NaOH, and the final volume of the mixture brought to 10 mL by addition of Milli-Q water. The mixture was allowed to incubate for 2 minutes at 37°C, under constant agitation. Since the test sample was liquid, simulated mastication or addition of human salivary α-amylase (EC 3.2.1.1) were not required.

Gastric Phase

The mixture resulting from the oral phase was carried out to the gastric phase by adding 8 mL of Simulated Gastric Fluid (KCl, 6.9 mM; KH_2PO_4, 0.9 mM; $NaHCO_3$, 25 mM; NaCl, 47.2 mM; $MgCl_2(H_2O)_6$, 0.12 mM; $NH_4(CO_3)_2$, 0.5 mM; $CaCl_2(H_2O)_2$, 0.15 mM; HCl, 15.6 mM; pH 3) and 1 mL of pepsin (EC 3.4.23.1) from porcine gastric mucosa (Sigma, USA). One molar HCl was added until pH 3 and water was added to bring the mixture to a final volume of 20 mL. The gastric digestion step was conducted for 2 hours at 37°C, under constant agitation.

Small Intestine Phase

Before the small intestine phase was started, a 5 mL sample of the gastric digestion mixture were collected to HPLC analysis. The enzymatic reaction was stopped by the addition of 6 M NaOH and the sample stored at -20°C. To the remaining volume of gastric digestion mixture were added 7 mL of Simulated Intestinal Fluid (KCl, 6.8 mM; KH_2PO_4, 0.8 mM; $NaHCO_3$, 85 mM; NaCl, 38.4 mM; $MgCl_2(H_2O)_6$, 0.33 mM; $CaCl_2(H_2O)_2$, 0.6 mM; HCl, 8.4 mM; pH 7), 1.88 mL of bile extract porcine (Sigma, USA) and 5 mL pancreatin from porcine pancreas (Sigma-Aldrich, USA). Finally, the mixture was brought to pH 7 by addition of 6 M NaOH and Milli-Q water added to yield a final volume of 30 mL. Incubation of the final intestinal digestion mixture occurred for 2 hours at 37°C, under constant agitation.

Further enzymatic action on the intestinal digestion sample was stopped through the addition of 10 µL of Pefabloc® (Sigma-Aldrich, USA) per milliliter of digested sample. The mixture was transferred to Amicon® Ultra-4 Centrifugal Filter Units (Merck Millipore, Germany) and centrifuged (Mikro 220R, Hettich, Germany) at the maximal velocity, for 40 minutes. All collected samples were stored at -20°C until further use.

2.5. Cell-Based Assays

2.5.1. Cell Culture

Human colon adenocarcinoma Caco-2 cells were obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (Braunschweig, Germany) and grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS). The stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO_2 humidified atmosphere. The cultured cells were split once or twice a week and their growth monitored daily. Cell culture medium and supplements were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK).

2.5.2. Cytotoxicity Assay

Cytotoxicity evaluation for all samples was performed using differentiated Caco-2 cells. Caco-2 cells are utilized as a model of the intestinal barrier because in long –term culture the cells suffer a spontaneous differentiation and polarization that leads to the formation of monolayers, expressing several functional and morphologic characteristics of mature enterocytes [101].

The procedure was done as described by Serra *et al.* [102] with some modifications. Cells were seeded in 96-well culture plates at a density of 2 x 10^4 cells/well, and the medium changed every 48 hours. The cells were allowed to grow for 8 days, until confluence and differentiation were reached. White wine samples in appropriate dilutions, were diluted in culture medium (RPMI 1640, supplemented with 0.5% FBS) and added to the wells. Control cells were carried out with only culture medium or in a mixture of the tested samples solvent and medium. Cells and white wine samples were incubated for 1 and 4 hours in a 5% CO_2 humidified atmosphere at 37ºC. Cytotoxicity determination was performed using Cell Titter® AQueous One Solution Cell Proliferation Assay (Promega, USA). The viability reagent was diluted according to the manufacturer information and incubated for 3 hours at 37°C under a 5% CO_2 humidified atmosphere. After incubation, absorbance was measured in a spectrophotometer (Spark® 10M, Tecan, Switzerland) at 490 nm. Results were expressed as viability, the percentage of living cells determined relatively to the control, and presented as means of triplicates ± standard deviation. Dose-response curves were plotted and IC_{50} values calculated using non-linear regression, with GraphPad Prism® (version 6.01, GraphPad Software Inc., USA).

2.5.3. Glucose Transport Measurements

Culture of Caco-2 cells on permeable supports allowed the cells to grow in an environment that better reproduces the steric conditions existing in the human small intestine, whereby, nutrients and ions have access to both sides of the monolayer, the apical side (intestinal lumen) and the basolateral side (blood stream). These conditions lead to a morphological and functional improved differentiation, such as the establishment of a trans-epithelial electrical resistance (TEER), that was used to monitor the integrity of the cell monolayer. After 20 days in culture, Caco-2 cells express several features of the mature small intestine enterocyte. Expression of sugar transporters (SGLT1, GLUT2 and GLUT5), as well amino acids, vitamins and other nutrients, have been identified [101].

Caco-2 cells were seeded at 2.24 x 10^5 cells/mL density in 12 mm Transwell® polycarbonate inserts (Corning®, USA). The cells were allowed to reach confluence and were cultured for 21 days under a 5% CO_2 humidified atmosphere, at 37°C. The medium (RPMI 1640, 10% FBS and 1% PS) was changed every 48 hours and the differentiation and integrity of the cell monolayer

assessed by TEER measurements. Inserts with TEER values exceeding 500 Ω were used in the experiments.

Glucose transport measurements were determined according to the method described by Manzano *et al.* [73]. After 21 days of culture, cell medium was carefully discarded from apical and basal compartments and both compartments were washed twice with HEPES buffer solution A (HEPES, 20 mM; NaCl, 137 mM; KCl, 4.7 mM; $MgSO_4$, 1.2; $CaCl_2$, 1.8 mM; pH 7.4). Then, HEPES buffer solution A was discarded from the apical compartment and 500 µL of the test solutions dissolved in solution B (HEPES buffer solution A containing 1 mM glucose (Sigma, USA) and D-[U-^{14}C] glucose 0.5 µCi/mL) were added. The basal solutions were HEPES buffer solution A. The plate was incubated at 37°C under a 5% CO_2 humidified atmosphere for 30 minutes. After the incubation, TEER measurements were taken in order to assess the integrity of the cell monolayers and solutions from both compartments were collected for scintillation counting. Membranes were washed twice with ice-cold 0.01 M PBS (Sigma-Aldrich, USA) to stop glucose uptake. Afterwards, cells were lysed by the addition of 500 µL of 0.1 M NaOH. Cells and base were incubated for 10 minutes at room temperature and aliquots were removed for scintillation counting and protein quantification.

The detection of the radio-labeled D-[U-^{14}C] glucose was performed by addition of 400 µL of each collected sample to 4 mL of scintillation liquid (Ecoscint XR, National Diagnostics, USA) and samples were analyzed by scintillation counting using a Beckman LS 6500 Scintillation System (Beckman Coulter Inc., USA).

The same protocol was followed to investigate the glucose transport under sodium-free conditions. The HEPES buffer solution A was modified so that the 137 mM NaCl were replaced with 137 mM KCl and adjusted to pH 7.4 with 10 M KOH.

The amount of glucose (mM) in each compartment was determined using glucose as an external standard for the calibration curve (0 – 1 mM). Dose-response curves were plotted and IC_{50} values calculated using non-linear regression with GraphPad Prism® (version 6.01, GraphPad Software Inc., USA).

The results were expressed as means of at least duplicates of mg of glucose ± standard deviation.

2.5.4. Total Protein Content

Total concentration of protein extracted from Transwell® inserts was determined by the Bradford method [103]. In a 96-well microplate 5 µL of each sample were added to 250 µL of Bradford reagent (Sigma-Aldrich, USA). The plate was allowed to incubate at room temperature for 30 minutes in an orbital microplate shaker (Orbit™ P4 Digital Shaker, Labnet, USA). Absorbance at 595 nm of the samples was measured in a microplate spectrophotometer (Spark® 10M, Tecan, Switzerland). Bovine serum albumin (Sigma, USA) was used as an external standard for the calibration curve (0 – 1.2 mg/mL). The results were expressed as means of triplicates (mg protein/mL) ± standard deviation.

3. Results and Discussion

The search of dietary natural compounds that can diminish post-prandial hyperglycemia peaks is of great interest. In this area, wines are very promising beverages due to their rich composition in polyphenolic compounds. Red wine has been extensively studied, existing some reports on its anti-diabetic potential. However, in white wine these health-promoting effects were not explored much.

The main aim of this thesis was to evaluate the potential anti-hyperglycemic effect of white wines using an *in vitro* approach. For this purpose, the capacity of wines in inhibiting carbohydrates digestion enzymes (Part 1) and intestinal glucose transporters (GLUT2 and SGLT1) (Part 2) was studied using *in vitro* assays and cellular models.

3.1. Part 1: White Wine Characterization and Evaluation of Carbohydrate Digestion Inhibitory Potential

In a previous work of the host lab focused on "White Wine and Health", the phytochemical content and antioxidant capacity of 4 white wines was evaluated. The results listed in table 3.1 are from the project report [104] and were the basis of the work of this master thesis. First, the antioxidant and phytochemical characterization of white wines was accomplished, and the results are summarized in tables 3.1 and 3.2.

Table 3.1: Phytochemical and antioxidant characterization of the samples. The results are expressed as mean ± SD. Results from the report of the project "White Wine and Health".

	TPC[1] (mg GAE/L)	ORAC[2] (µM TEAC)	HORAC[3] (µM CAE)	CAA[4] (µM QE)
Aveleda Douro Doc (2011)	283 ± 33	8226 ± 843	3669 ± 311	447
Quinta da Aveleda (2011)	250 ± 30	7017 ± 811	3556 ± 395	464
Aveleda Alvarinho (2011)	290 ± 34	6408 ± 562	4746 ± 272	424
Conventual (2011)	279 ± 33	6986 ± 504	4014 ± 270	356

1- Total phenolic content (TPC) determined using *Folin-Ciocalteau* method. The results are expressed as mg of gallic acid /L of wine. **2-** Rescue capability of peroxide radicals. The results are expressed as µmol of trolox equivalents /L of wine. **3-** Inhibition capability of hydroxide radicals. The results are expressed as µmol of caffeic acid equivalents /L of wine. **4-** Cellular antioxidant capacity: rescue capability of reactive species at cellular level using Caco-2 cell model and AAPH as oxidant agent. The results are expressed as µmol of quercetin equivalents /L. The method has an associated error inferior of 15%.

In terms of antioxidant capacity (Table 3.1), no major differences were observed between the four wines, but the samples from Douro region present higher levels of antioxidant capacity: Aveleda Douro Doc yielded the highest ORAC value and Aveleda Alvarinho the highest HORAC value; the highest value of cellular antioxidant capacity is from Quinta da Aveleda. The total phenolic content (TPC) was similar for all tested samples, but again a Douro wine presented the highest phenolic content: Aveleda Alvarinho. The total phenolic content of white wines was in agreement

with the literature, and about 10 time less than the reported phenolic content for red wine [105,106]. Despite there being no differences between the total phenolic content of the analyzed white wines, the antioxidant activity of the samples differs and this result could be related with the presence of a specific compound and not with the total sum of compounds.

In order to analyze the phenolic composition of each tested sample, LC-MS/MS was performed for the detection and quantification of specific phenolic compounds by using calibration curves of relevant standards. The quantification results are summarized in table 3.2.

Table 3.2: Identification and quantification of phenolic compounds in the four white wine samples using LC-MS/MS with electrospray ionization. The results are expressed as mean ± SD of duplicate.

		Wine Sample			
		Aveleda Douro Doc (2011)	**Quinta da Aveleda (2011)**	**Aveleda Alvarinho (2011)**	**Conventual (2011)**
Identified Compounds (mg/L)	**Caffeic Acid**	1.3 ± 0.14	1.35 ± 0.07	2.05 ± 0.07	1.25 ± 0.07
	***p*-Coumaric Acid**	0.55 ± 0.07	0.55 ± 0.07	1.15 ± 0.07	0.65 ± 0.07
	Ferulic Acid	NI	NI	NI	< LOQ
	Gallic Acid	3.4 ± 0.14	3.95 ± 0.5	5.25 ± 0.35	2.55 ± 0.21
	Rutin	< LOQ	< LOQ	< LOQ	< LOQ
	Procyanidin B1	< LOQ	1 ± 0.14	2.5 ± 0.14	< LOQ
	Catechin	< LOQ	0.84 ±0.09	1.1 ± 0.14	NI
	Gallocatechin	NI	NI	0.6 ± 0.0	0.9 ± 0.0
	Coutaric Acid	8.55 ± 0.5	3.6 ± 0.14	6.7 ± 0.0	4.3 ±0.07
	Fertaric Acid	13.2 ± 0.07	16.2 ± 0.0	22.3 ± 1.5	24.5 ± 0.42
	Caftaric Acid	NI	NI	NI	NI

NI, not identified; LOQ, limit of quantification

From the results presented in table 3.2 is evident that the identified compounds were mostly phenolic acids, more precisely hydroxycinnamic acids. This is in agreement with the literature, since hydroxycinnamic acids are identified as the phenolic compounds existing at higher concentrations in white wine [84]. Caffeic and *p*-coumaric acids were identified in all samples as simple acids, as well as coutaric acid. Ferulic acid was not identified in Douro region wines but was identified below the quantification limit in Conventual. However, fertaric acid (the ester of ferulic and tartaric acids) was identified and quantified in all wines and the highest concentration was obtained for Aveleda Alvarinho. Among all samples Aveleda Alvarinho was the wine that presented more variety of phenolic compounds at higher concentrations, particularly *p*-coumaric acid, gallic acid, procyadinidin B1 and catechin. Conventual presented the highest concentration of fertaric acid and the lowest of gallic acid. Aveleda Douro Doc was the sample that presented

the lowest concentrations and less variety of the different phenolic acids identified, although it had the highest amount of coutaric acid. The observed differences in phenolic composition between the four white wines were probably derived from the different types of grapes present in each wine (section 2.1).

The α-amylase inhibitory potential of white wines was evaluated. Inhibition of pancreatic α-amylase results in a decrease of the starch hydrolysis, diminishing the amount of oligosaccharides in the small intestine lumen that are substrates of brush border α-glucosidases, therefore preventing the absorption of simple sugars. The results are summarized in Figure 3.1. In this work, acarbose was used as a positive control and the results showed that this drug inhibited α-amylase in a dose-dependent manner, yielding an IC_{50} value of 35.56 ± 1.37 mg/L (≈ 55.08 μM) (Appendix A, figure 6.1), in agreement with the literature [93,107].

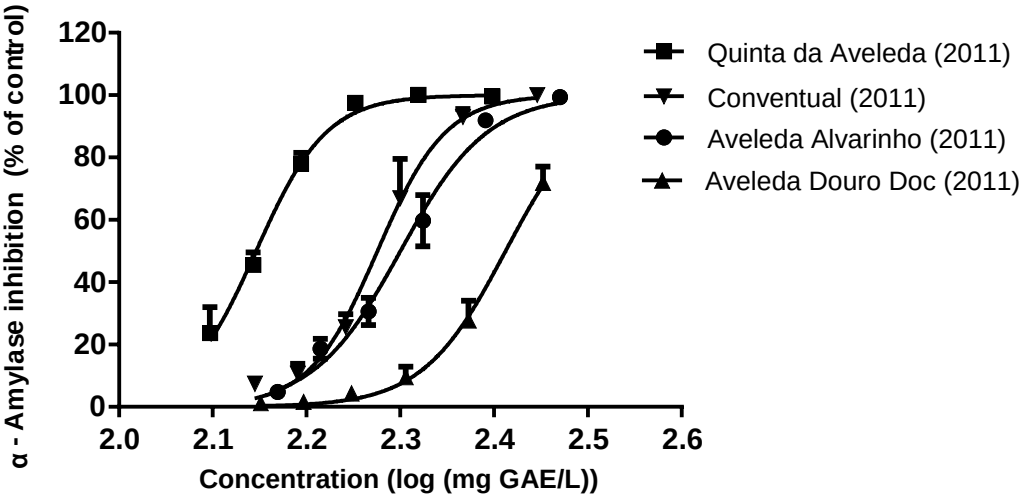

Figure 3.1: Dose-response curves of Quinta da Aveleda (2011), Conventual (2011), Aveleda Alvarinho (2011) and Aveleda Douro Doc (2011). Results are expressed as mean ± SD of three independent experiments performed in triplicate.

All four white wines were efficient inhibitors of α-amylase, in a dose-dependent manner (Figure 3.1). The IC_{50} values were determined from their correspondent dose-response curve using non-linear regression and are presented in Table 3.3.

Table 3.3: IC_{50} values (mg GAE/L) of the four white wine samples obtained from the respective dose-response curves presented in Figure 3.1. Results are shown as mean ± SD of three independent experiments performed in triplicate.

Sample	IC_{50} (mg GAE/L)
Aveleda Alvarinho (2011)	199.5 ± 3.11
Aveleda Douro Doc (2011)	258.3 ± 1.94
Quinta da Aveleda (2011)	140 ± 1.73
Conventual (2011)	188.6 ± 2.09

The results showed that, when compared with acarbose, the IC_{50} values of all wines were higher, indicating lower inhibition capacity than the drug. Even so, the samples demonstrated to have a high inhibitory potential, with Quinta da Aveleda showing the most potent α-amylase inhibition, and Aveleda Douro Doc the weakest. Studies with white wine pomace had demonstrated that white grape skin residues were found to inhibit α-amylase with an equal or higher efficacy than acarbose, with IC_{50} values ranging from 12.5 to 27.4 mg GAE/L, about 10 times more potent than the values determined for white wine [93].

The α-glucosidase inhibitory potential of the four white wines was evaluated in rat small intestine α-glucosidases. Studies have demonstrated the advantages of using α-glucosidases of mammalian origin since they are biologically more relevant compared to purified yeast α-glucosidases, mimicking more accurately the *in vivo* processes [92]. However, one of the main groups of macromolecules existing in white wine are polysaccharides [108]. Since some of these types of sugars are substrates to α-glucosidases, in order to eliminate their interference on the enzyme activity, phenolic fractions were collected in methanol by SPE. After the phenolic extraction, the samples were concentrated and all wines were evaluated at the same concentration factors (CF). Different concentrations of the wines were tested, namely CF 2.5 and CF 5. However, in this case, it was not possible to determine the IC_{50} value due to color interference of the samples with the enzymatic inhibition method used. Even using a control only to account for the color interference it was not possible to determine the percentage inhibitions for concentrations superior to CF 5 (data not shown). Therefore, percentage inhibitions at CF 5 and CF 2.5 were determined and presented in Figure 3.2. Acarbose was used as a positive control and its dose-response curve was determined (Appendix A, Figure 6.2), yielding an IC_{50} value of 31.9 ± 9.65 mg/L (≈ 49.4 µM) which is similar to other reports [52,109,110].

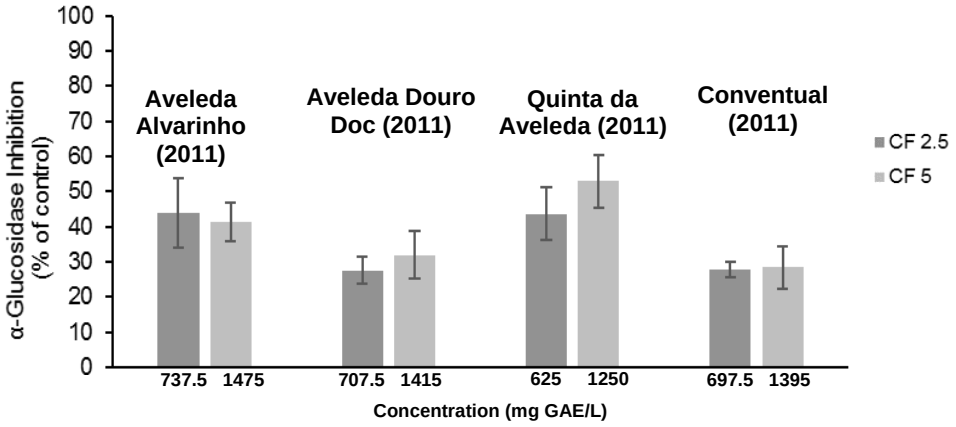

Figure 3.2: Inhibitory effect of the tested white wines in rat small intestinal α-glucosidase. Results are expressed as mean ± SD of three independent experiments performed in triplicate.

Among all samples, Quinta da Aveleda, which at the highest concentration tested (CF 5) reached about 50% inhibition, and Aveleda Alvarinho were the most potent inhibitors. These results are very low in comparison with the IC_{50} values found for white grape skins, whose values varied from 3.9 mg GAE/L to 93.1 mg GAE/L, much lower than the polyphenolic concentrations tested [93]. In another study, black tea was found to be a very potent α-glucosidase inhibitor, with IC_{50} values 25 times higher than those determined for white grape skins [110]. It is noteworthy that the highest inhibitory potential observed for Quinta da Aveleda was not related with a higher polyphenolic concentration, since the tested concentration was the lowest (1250 mg GAE/L) of all four wines at CF 5.

Aiming at evaluating if white wine phenolics can act synergistically with acarbose in inhibiting α-glucosidase, three different concentrations of acarbose (1000 mg/L: 75% inhibition; 62.5 mg/L: 50% inhibition; 1.95 mg/L: 35% inhibition) and one of each wine (CF 2.5) were tested and the results summarized in Figure 3.3.

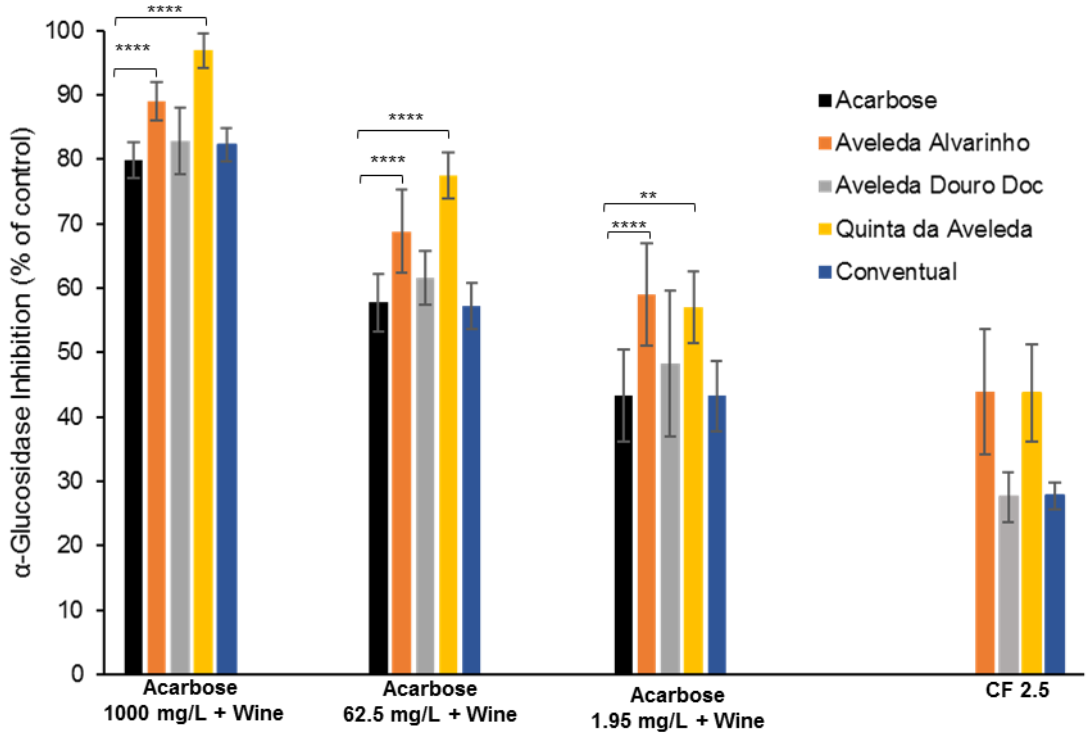

Figure 3.3: Inhibitory effect of combining acarbose and white wines in rat small intestinal α-glucosidase. The four wines were tested at the following concentrations: Aveleda Alvarinho, 737.5 mg GAE/L; Aveleda Douro Doc, 707.5 mg GAE/L; Quinta da Aveleda, 625 mg GAE/L; Conventual, 697.5 mg GAE/L. The results are expressed as means ± SD of three independent experiments performed in duplicate. Significant differences from acarbose are expressed with asterisks (ns $P > 0.05$; **$P < 0.01$ and ****$P < 0.0001$) by one-way ANOVA.

Aveleda Alvarinho (2011) and Quinta da Aveleda (2011) in combination with acarbose showed significant ($P < 0.01$ and $P < 0.0001$) higher α-glucosidase inhibition than the drug alone. For the other wines, no improvements on the α-glucosidase inhibitory effect were observed when combined with the drug. The differences in response between wines could be related with their phenolic composition. Aveleda Alvarinho and Quinta da Aveleda could contain phenolic compounds that present synergy with acarbose.

In order to identify the bioactive compounds responsible for the α-glucosidase inhibition and for the effect observed in combination with acarbose, pure phenolic standards were tested. Aveleda Alvarinho (2011) and Quinta da Aveleda (2011) contained approximately the same phenolic compounds that were reported in the literature as potential α-glucosidase inhibitors or whose structures suggested that they were good candidates. Thus, *p*-coumaric acid, caffeic acid, catechin and gallic acid were tested for their inhibitory potential. All compounds were tested in the same concentrations found in Aveleda Alvarinho. The results are presented in Figure 3.4 and the concentrations of the pure compounds tested summarized in Table 3.4. Aveleda Alvarinho inhibition percentage was used to assess the dependence with the pure compounds inhibition.

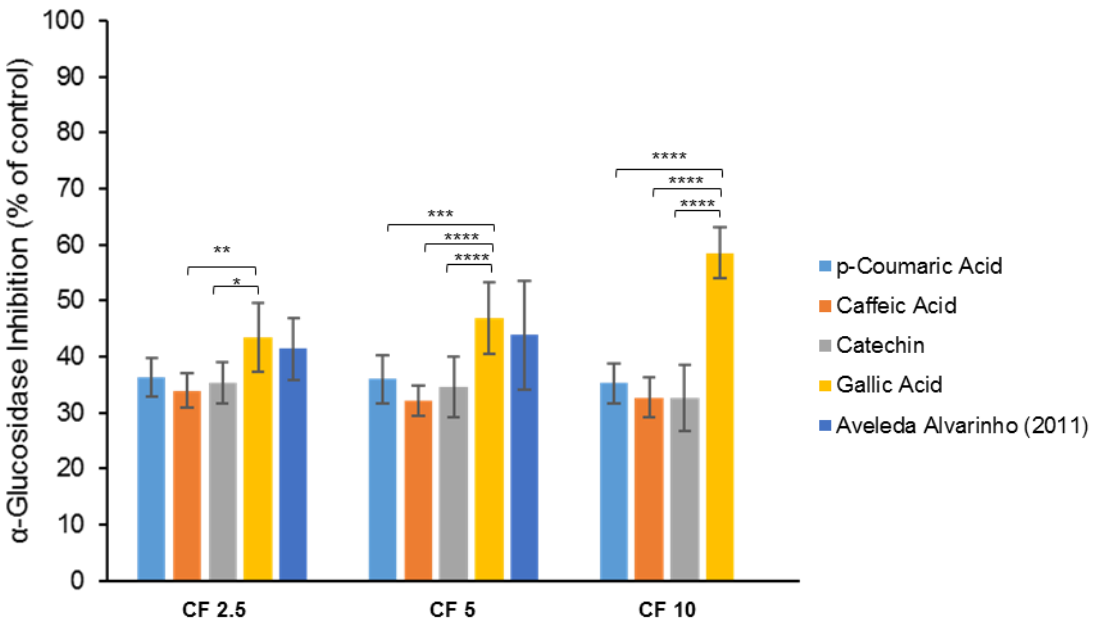

Figure 3.4: Inhibitory effect of pure phenolic compounds in rat small intestinal α-glucosidase, at three different concentrations of Aveleda Alvarinho wine CF: 2.5, 5 and 10. The concentrations of each tested compound are described in Table 3.4. Aveleda Alvarinho (2011) was tested at 737.5 mg GAE/L (CF 2.5) and 1475 mg GAE/L (CF 5). The results are expressed as means ± SD of three independent experiments performed in triplicate. Significant differences from gallic acid are expressed with asterisks (ns $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ and ****$P < 0.0001$) by one-way ANOVA. Due to the increasing of color with concentration, it was not possible to determine a result for the wine at CF 10.

Table 3.4: Tested concentrations of the pure compounds at the different concentration factors of Aveleda Alvarinho (2011) wine.

	Concentration (mg/L)		
	CF 2.5	**CF 5**	**CF 10**
***p*-Coumaric Acid**	3	6	12
Caffeic Acid	5.3	10.5	21
Catechin	2.8	5.5	11
Gallic Acid	13.3	26.5	53

The results showed that gallic acid was the only pure phenolic compound that significantly inhibited α-glucosidase in a dose-dependent manner ($P < 0.0001$), reaching 60% enzymatic inhibition in the higher concentration tested. The other compounds, namely *p*-coumaric acid, caffeic acid and catechin, exerted approximately 35% inhibition at all three concentration factors tested. In order to understand if any of the tested compounds was responsible for the wine inhibition percentage previously observed, obtained inhibitions from the pure compounds were compared with Aveleda Alvarinho at concentration factors 2.5 and 5. Observing Figure 3.4 it appears that the effect exerted by gallic acid is very similar to the inhibition that Aveleda Alvarinho exerts in the enzyme at CF 2.5 and 5, suggesting that it could be the bioactive compound responsible of most of the inhibitory potential that the wine demonstrated. A statistical analysis was done in order to determine if gallic acid produced a significant different inhibition from *p*-coumaric acid, caffeic acid and catechin, and if it were similar to the one produced by the wine. The results showed that in fact gallic acid did not produced a significantly different inhibition than

the wine, at each of the concentrations tested, and it was significantly different from the other pure compounds tested. Comparison between the remaining phenolic compounds revealed no statistically significant differences existed between them (data not shown). Thus, since gallic acid revealed to be a promising bioactive compound in α-glucosidase inhibition, a dose-response curve was performed and its IC_{50} was calculated (Figure 3.5).

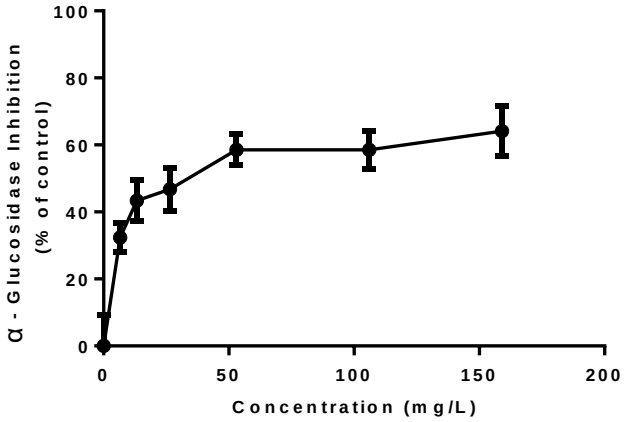

Figure 3.5: Dose-response curve of gallic acid in inhibiting α-glucosidase. Results are expressed as mean ± SD of three independent experiments performed in triplicate.

Figure 3.5 showed that gallic acid produced a dose-dependent inhibition of the enzyme, yielding an IC_{50} of 32.28 ± 9.65 mg/L (≈ 195.6 µM).These results support the observations made by other researchers that studied gallic acid inhibitory activity in different models: yeast α-glucosidase, brush border membranes from rat and maltase at the apical side of Caco-2 cell model [111,112].

Gallic acid was then combined with acarbose in order to evaluate if this phenolic compound present in white wine could act synergistically with the drug, improving the inhibition of this carbohydrate-degrading enzyme. For this, the combination of both compounds at the same concentration (1:1 ratio) was tested and the dose-response curve determined. The obtained results are in Figure 3.6.

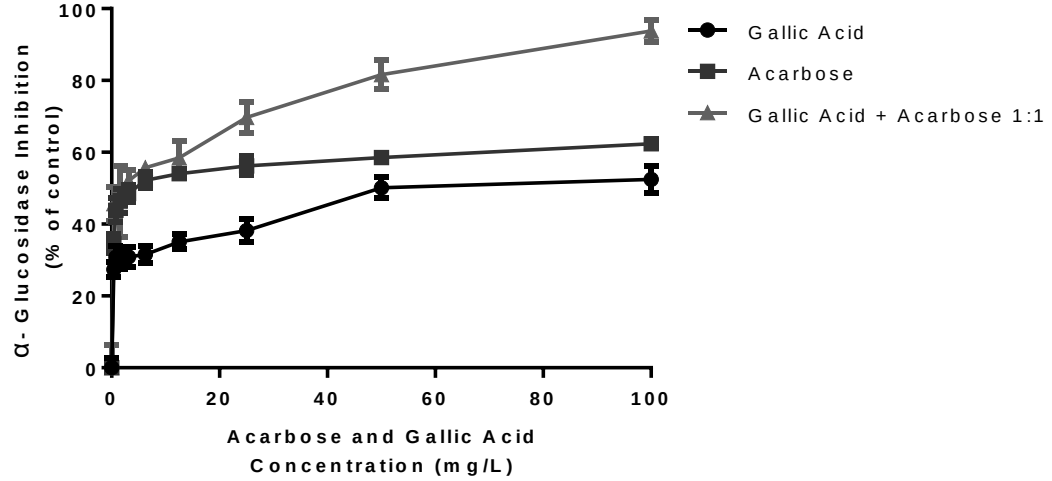

Figure 3.6: Dose-response curves of gallic acid, acarbose and of gallic acid/acarbose combination in 1:1 ratio. Results are expressed as mean ± SD of three independent experiments performed in triplicate.

The results in Figure 3.6 and the IC_{50} above demonstrated that gallic acid is a much weaker α-glucosidase inhibitor than acarbose. When tested in the same proportion, gallic acid and acarbose, produced a much stronger inhibition than the observed in the isolated compounds, reaching approximately 100% inhibition in the highest concentration tested (100 mg/L). In order to determine if the observed combined effect of gallic acid and acarbose was synergy, additive effect or antagonism, the combination indexes (CI) for each tested concentration were calculated through Chou-Talalay method, using CompuSyn® software. The combination index equation allows the quantitative determination of the interaction, that can be synergism (CI < 1), additive effect (CI = 1) or antagonism (CI >1) [99]. The results from the software analysis of the tested combinations are in Appendix B. Overall, the resulting effect of the combination of gallic acid/acarbose was synergy (CI < 1) as expected. In fact, the presence of a very strong synergy between the two compounds was observed (CI < 0.1) [113].

Taken together, all these results indicated that although white wines presented lower phenolic content than red wines, they contain a wide variety of phenolic acids and are able to inhibit carbohydrate-digestion enzymes *in vitro*. Among all samples tested, Aveleda Alvarinho (2011) demonstrated to be the most promising white wine tested. This wine presented the highest concentration of total phenolic compounds and the most variety of different phenolic acids in its composition, as well as displayed a strong α-glucosidase inhibition and synergy with the drug acarbose. Importantly, this wine contained the highest gallic acid concentration, a phenolic compound with α-glucosidase inhibitory capacity and synergistic effect in combination with the drug.

3.2. Part 2: White wine *in vitro* Digestion and Evaluation of Glucose Absorption Inhibitory Potential

Aveleda Alvarinho was the selected wine for the evaluation of glucose inhibitory potential due to the results previously obtained, which demonstrated the higher phenolic content of the wine, as well as its capacity to inhibit α-glucosidase, demonstrating synergy with acarbose. For this study, a new sample of Aveleda Alvarinho from a more recent harvest (2014) was analyzed. Aveleda Alvarinho (2014) was characterized in terms of phenolic profile. Quantification of total phenolic content was assessed by the Folin-Ciocaulteu method and a concentration of 305.6 ± 25.6 mg GAE/L was determined, which is in agreement with the total phenolic content of Aveleda Alvarinho (2011) described in section 3.1 and with the total phenolic content reported in the literature for different white wines [105,114]. The phenolic profile of the wine was analyzed by HPLC-DAD at 280 nm, 320 nm and 360 nm, which are the characteristic wavelengths of phenolic compounds, hydroxycinnamic acids and flavonoids, respectively, aiming at the identification and quantification of phenolic compounds of interest. Figure 3.7 presents the Aveleda Alvarinho (2014) phenolic profile.

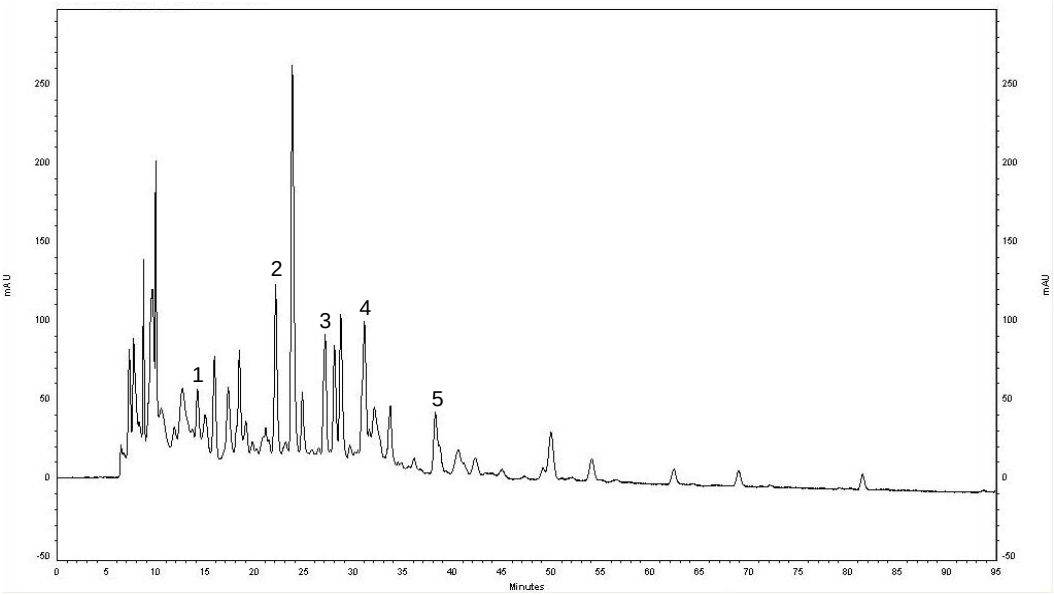

Figure 3.7: Phenolic profile of Aveleda Alvarinho (2014) recorded at 280 nm. Legend: 1– gallic acid, 2– neochlorogenic acid, 3– epicatechin, 4– caffeic acid, 5– *p*-coumaric acid.

As presented in Figure 3.7, gallic acid, neochlorogenic acid, epicatechin, caffeic acid and *p*-coumaric acid, were identified. Through the analysis of the chromatograms the presence of chlorogenic acid (isomer of neochlorogenic acid) and *trans*-piceid were observed in the sample. Both compounds were reported in the literature as existing in the white wine phenolic composition [115,116]. In contrast with other phytochemical studies of white wines, ferulic acid and resveratrol were not identified in the sample [117,118]. In fact, these relevant bioactive compounds associated to a decrease in intestinal glucose absorption through SGLT1 transporter, were also not present in Aveleda Alvarinho from 2011 nor in the other white wines analyzed by mass spectrometry (section 3.1) [68,77].

Quantification of the identified compounds was achieved using calibration curves of pure standards and the results are presented in Table 3.5.

Table 3.5: Concentration of the identified phenolic compounds in Aveleda Alvarinho (2014) by HPLC-DAD.

Compound	Concentration (mg/L)
Gallic Acid	3.49
Neochlorogenic Acid	21.49
Epicatechin	32.67
Caffeic Acid	2.66
***p*-Coumaric Acid**	0.54

Results showed that the wine is very rich in neochlorogenic acid and epicatechin. Although it was reported in the literature that these compounds were present in the phenolic composition of white wines, the concentrations found were usually lower, 2.9 mg/L for neochlorogenic acid and 0.9 mg/L for epicatechin [119]. However, one study with French white wine reported a concentration of epicatechin of 33.8 mg/L, similar to the result in Table 3.5 [118]. In future, further analysis should be done by HPLC-MS analysis, in order to confirm the present results and also quantify other phenolic compounds of interest that were detected in the HPLC-DAD-UV chromatograms. The concentrations of gallic acid and *p*-coumaric acid were lower in comparison with the results of Aveleda Alvarinho (2011) presented in section 3.1, although in agreement with the literature [105,120].

Since gastrointestinal digestion can change the phenolic composition of foods, altering phenolic bioaccessibility, Aveleda Alvarinho (2014) was subjected to a standardized static *in vitro* simulated digestion. In fact, gastrointestinal digestion involves changes in pH and the activity of proteolytic enzymes that could affect the phenolic composition of white wine. The digestion process was performed in four independent experiments and samples of the gastric and intestinal steps were collected and analyzed by HPLC-DAD. The comparison of the phenolic profiles of the four replicates is presented in Figure 3.8.

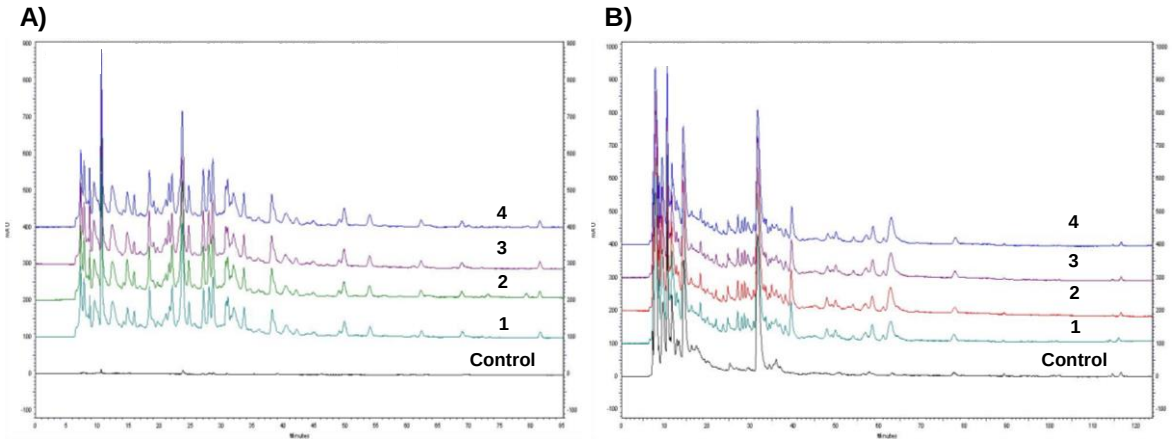

Figure 3.8: Phenolic profiles of the four in vitro digestion replicates (1, 2, 3, 4) and digestion control (performed with Milli-Q water instead of wine) of Aveleda Alvarinho (2014), collected in the gastric phase (A) and the small intestinal phase (B). The results were obtained by HPLC-DAD and recorded at 280 nm.

All replicates were characterized in terms of total phenolic content by the Folin-Ciocalteau method, in each digestion step, and the results are summarized in Figure 3.9.

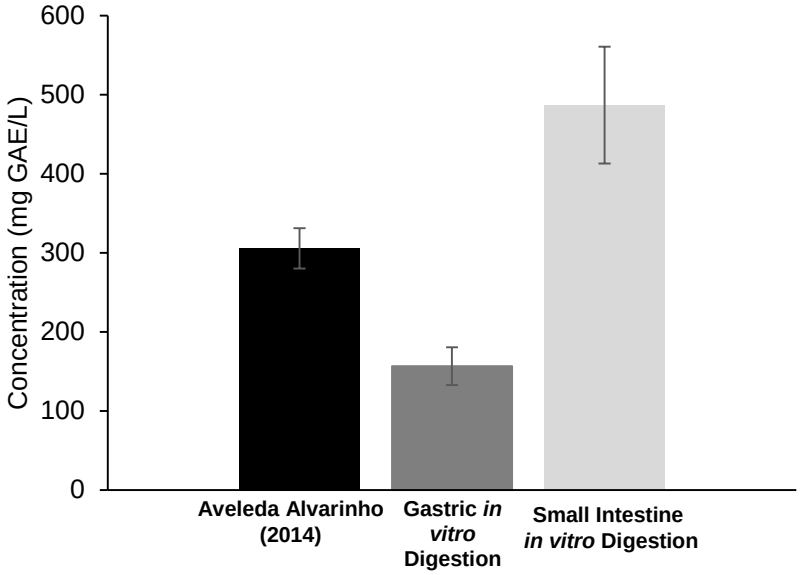

Figure 3.9: Total phenolic content of gastric and small intestinal white wine after simulated digestion. The results are expressed as mg of gallic acid equivalents/L and shown as mean of four independent experiments ± SD.

Results from Figures 3.8 and 3.9 showed that the four different wine digestion samples presented high reproducibility. It is important to mention that during the digestion process the phenolic content of the wine suffered changes. Results from Figure 3.9 showed that after the gastric step the phenolic content lowered, although a marked increase is observed after the intestinal step relatively to the initial wine. The phenolic profiles and the phenolic content of Aveleda Alvarinho (2014) and the gastric and intestinal fractions are compared in Figure 3.10. Analysis of the chromatographic profile of the intestinal fraction in Figure 3.10 revealed the appearance of new peaks, some with considerable areas, such as the peak at 63 minutes. This increase in the phenolic content was probably due to the contribution of these newly formed compounds. In solid matrices such as grape berries, an increase in phenolic content has been reported after *in vitro* digestion due to the extraction of phenolic compounds from the skin of the fruit in the gastric phase, which increases the amount of bioaccessible polyphenols during the digestion, although the same was not reported for beverages and juices [121,122]. However, it should be noted that this increase could be from interference from the newly formed compounds that could have higher redox potential than their precursors, thus interfering with the Folin-Ciocalteu method [123]. Further analysis is necessary in order to understand which metabolites are formed following the *in vitro* digestion process.

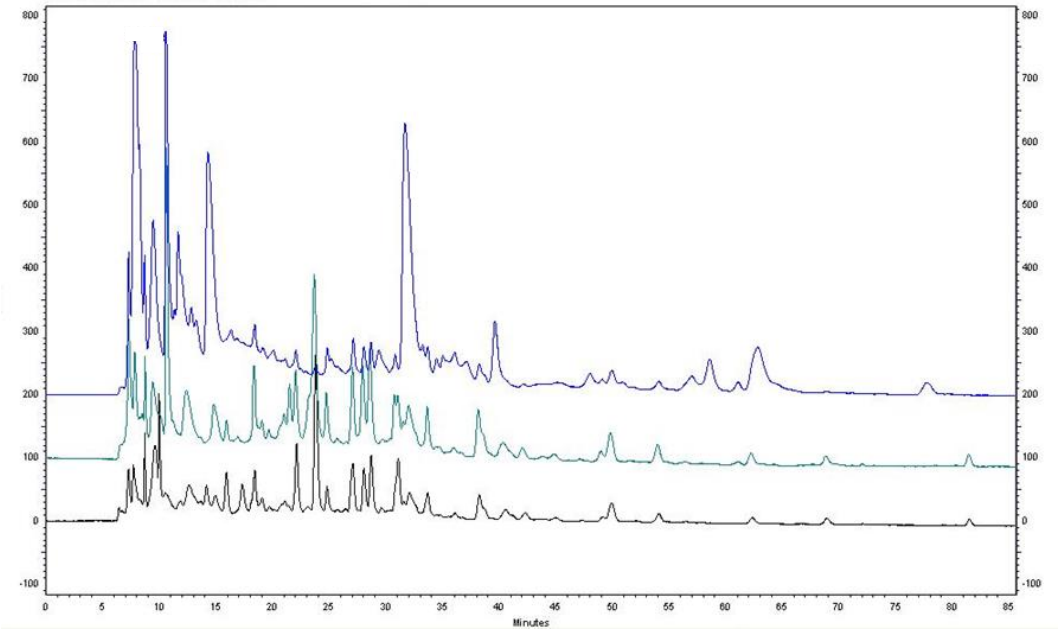

Figure 3.10: Phenolic profiles of Aveleda Alvarinho (2014), gastric fraction and small intestinal fraction recorded at 280 nm, obtained by HPLC-DAD.

The results presented in Figure 3.10 evidence changes between the two steps of the gastrointestinal process. Phenolic profiles of Aveleda Alvarinho (2014) and its gastric digested fractions were quite similar, although the intensity of most peaks was higher in the gastric sample. In the small intestinal fraction, there were significant alterations to the phenolic profile, since the intensity of some peaks decreased and new peaks appeared. Some peaks that appeared exclusively in the intestinal fraction were derived from solutions and enzymes used in the digestion process, as these peaks also appeared in the control of the experience (Figure 3.8).

Next, the identification and quantification of the main bioactive phenolic compounds of both gastric and intestinal fractions was carried out by HPLC-DAD and the results are presented in Figure 3.11 and Table 3.6.

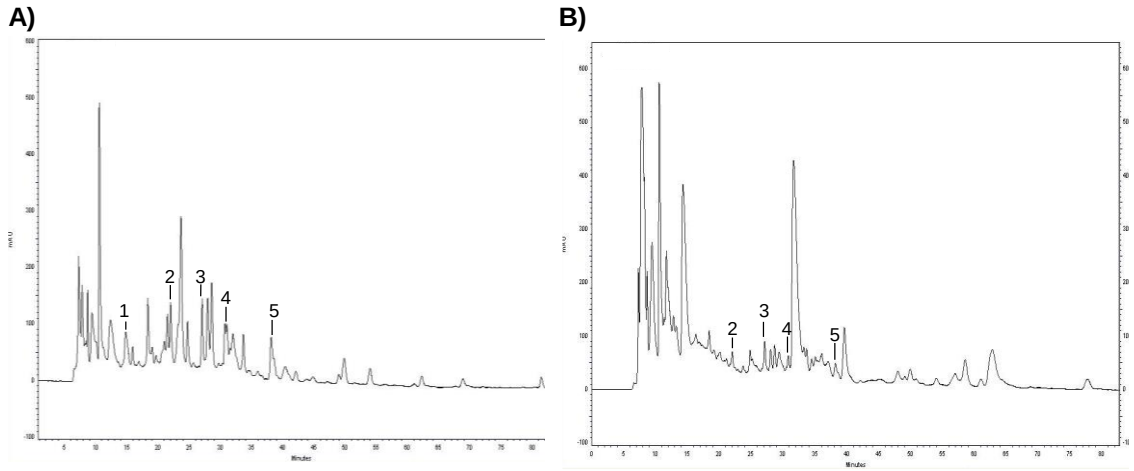

Figure 3.11: Phenolic profile of gastric (A) and small intestinal (B) fractions, recorded at 280 nm. Legend: 1– gallic acid, 2– neochlorogenic acid, 3– epicatechin, 4– caffeic acid, 5– *p*-coumaric acid.

The identified phenolic compounds were quantified through use of calibration curves of pure standards. The results are summarized in Table 3.6.

Table 3.6: Concentration of the identified phenolic compounds in the gastric and small intestinal digested fractions of Aveleda Alvarinho (2014) in replicate 3, by HPLC-DAD.

Compound	Concentration (mg/L)		
	Aveleda Alvarinho (2014)	Gastric *in vitro* digestion	Small Intestinal *in vitro* digestion
Gallic Acid	3.49	8.99	ND
Neochlorogenic Acid	21.49	22.98	4.66
Epicatechin	32.67	46.15	17.55
Caffeic Acid	2.66	3.35	< LOQ
***p*-Coumaric Acid**	0.54	2.45	< LOQ

ND, not detected; LOQ, limit of quantification

In the gastric fraction of digested white wine, gallic acid, neochlorogenic acid, epicatechin, caffeic acid and *p*-coumaric acid were identified and quantified. Chlorogenic acid and *trans*-piceid were both identified in the sample, whereas ferulic acid and resveratrol were not present. Neochlorogenic acid was the only phenolic compound that did not show significant differences regarding its initial concentration in the wine. The concentration of the remaining compounds increased after the treatment. Among all compounds, epicatechin and gallic acid showed the highest increase, followed by *p*-coumaric acid, whereas caffeic acid only suffered a slight increase in concentration. The stability of phenolic compounds under gastric acidic conditions has been studied. The increase in concentration of gallic acid, caffeic acid and *p*-coumaric acid after gastric digestion has been reported in the literature and is mostly due to the acidic gastric conditions [121,124]. Phenolic compounds from natural sources exist mainly as glycosides and esters, being hydrolyzed to free acids under acid conditions [125,126]. It is known that hydroxycinnamic acids in wine and grapes are found mostly in the form of esters (caftaric, coutaric and fertaric acids), and the increased concentration of caffeic and *p*-coumaric acids are probably due to their release from the esters [83,126]. Gallic acid also mostly exists in wine in complex polymeric structures, that under the gastric conditions could be broken, liberating gallic acid monomers, which contribute to the increase of gallic acid concentration after this digestion step [124,127]. Other authors have shown that epicatechin was stable under these conditions and that chlorogenic acid was not only stable but also preserved its initial concentration [128–130].

In this study, after the small intestine *in vitro* digestion step, neochlorogenic acid, epicatechin, caffeic acid and *p*-coumaric acid demonstrated marked concentration decreases. *Trans*-piceid was also found in the sample however the peak decreased in intensity. Caffeic and *p*-coumaric acids were identified, although their concentrations were below the limit of quantification. Similar decreases in phenolic acids concentration after *in vitro* digestion have been reported in the literature, because phenolic acids become unstable due to the mild alkaline conditions in the small intestine [121,128–130]. Gallic acid could not be identified in the sample due to the presence of other non-phenolic compounds derived from the gastric fluid that interfere with the analysis (retention time: 14.8 min). The peaks appeared in the beginning of the chromatogram with very high intensity, at the same time the gallic acid peak should appear. Results from the literature

reported a decrease in gallic acid concentration after intestinal digestion, which was expected due to its acidic nature [121,124]. Thus, further analysis should be done in order to determine the impact of intestinal digestion in gallic acid concentration.

The present results, as well as previous results from the literature, revealed that the phenolic compounds from white wine were stable under gastric digestion, promoting the release of some free acids. However, the compounds are highly sensitive to mild alkaline conditions, suggesting that a portion of these compounds must undergo structural transformations, giving rise to different compounds with different proprieties. Further testing and other analytical methods should be employed in order to assess the extension of the transformations, as well the identification and characterization of the new compounds. In the future, it would be of great interest to simulate the last step of intestinal digestion with gut microbiota, in order to evaluate the transformations that polyphenols suffer before absorption in the colon.

The effect of white wine and gastrointestinal digested white wine on intestinal glucose transport was then evaluated, using polarized Caco-2 cells. Caco-2 cell model has been used in this area to evaluate the effects of drugs and other compounds, such as phenolic compounds, in intestinal sugar absorption because of its high expression levels of both SGLT1 and GLUT2 transporters [131].

First of all, cytotoxicity of both wine and digested wine was assessed on Caco-2 cell monolayers. Samples and Caco-2 cells were incubated for one hour and the results are presented in Figure 3.12.

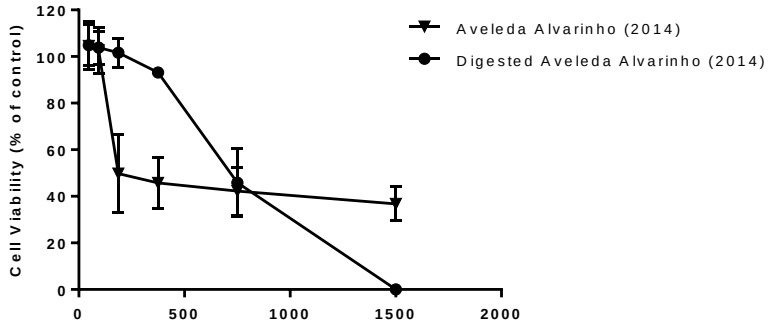

Figure 3.12: Cytotoxic evaluation of Aveleda Alvarinho (2014) and its digested fraction on Caco-2 cells. Incubation = 1 h; Results are expressed as mean ± SD of two independent experiments performed in duplicate.

The IC_{50} values for Aveleda Alvarinho (2014) and its digested fraction were determined. The results demonstrated that the wine was more toxic (IC_{50} 456.4 ± 141.5 mg GAE/L) than its digested fraction (IC_{50} 719.5 ± 5 mg GAE/L). The cytotoxic effect of Aveleda Alvarinho (2014) and digested fraction were also evaluated during a longer incubation time of 4 hours, however similar results were obtained (Appendix C).

The potential of white wine and the gastric fraction in inhibiting glucose transport across the small intestinal epithelium was then evaluated. Firstly, with the objective of evaluating glucose transport across the cell monolayer (from the apical to the basolateral side) as well as cellular uptake, promoted by glucose transporters, controls under sodium-dependent and sodium-free conditions were compared relatively to initial glucose mass as percentage (Figure 3.13).

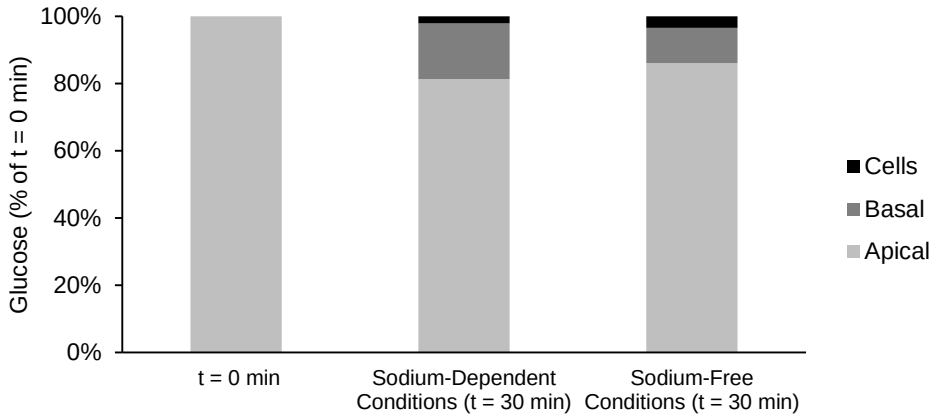

Figure 3.13: Distribution of glucose between apical side, basolateral side and cells after 30 minutes of incubation, as a percentage of initial glucose in apical side (t = 0 minutes). A single representative experiment is shown; however, results were similar for five independent experiments.

Under sodium-dependent conditions SGLT1 and GLUT2 were both activated, which reflected in more amount of glucose that was transported from the apical to the basolateral side. On the other hand, under sodium-free conditions only GLUT2 was operating and glucose was transported at a lower rate, although an increase of glucose uptake was observed (Figure 3.13). Similar results already reported demonstrated that, under sodium-free conditions, the glucose transport rate decreased due to inactivation of SGLT1 and therefore the glucose uptake observed is solely done by GLUT2 [132].

The study of wine and the inhibitory potential of its digested fraction was then carried out under sodium-dependent and sodium-free conditions. Different concentrations of wine and digested wine were tested according to polyphenol content, and dose-response curves were determined under both conditions (Figure 3.14). Known inhibitory compounds of SGLT1 and GLUT2 were used as positive controls of the experiments. Phloridzin (0.5 mM) was used under sodium-dependent conditions, inhibiting SGLT1 by 39.9 ± 3.9%. Phloretin (0.5 mM) inhibited GLUT2 under sodium-free conditions by 89.1 ± 1.8%.

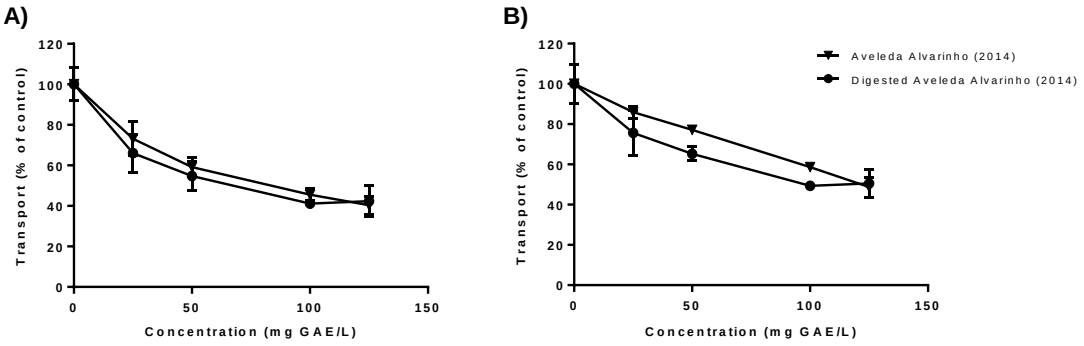

Figure 3.14: Effect of Aveleda Alvarinho (2014) and its gastrointestinal digested fraction on glucose transport across Caco-2 cells monolayers seeded in Transwell® inserts. Concentration of transported glucose was measured under sodium-dependent (A) and sodium-free (B) conditions. Results are expressed as mean ± SD of two independent experiments.

The inhibition of intestinal glucose absorption could result in a decrease in glucose concentration in plasma [73]. Both samples inhibited transported glucose in a dose-dependent manner under both conditions, allowing the determination of IC_{50} values, presented in Table 3.7.

Table 3.7: IC_{50} values (mg GAE/L) of Aveleda Alvarinho (2014) and gastrointestinal fraction, obtained from the respective dose-response curves, under sodium-dependent and sodium-free conditions. Results are expressed as mean ± SD of two independent experiments.

	IC_{50} (mg GAE/L)	
	Aveleda Alvarinho (2014)	**Digested Aveleda Alvarinho (2014)**
Sodium-Dependent Conditions (SGLT1 and GLUT2)	79.5 ± 2.6	67.5 ± 13.5
Sodium-Free Conditions (GLUT2)	126.7 ± 19.3	113.3 ± 31.8

Overall, the digested fraction of Aveleda Alvarinho (2014) displayed stronger glucose transport inhibition than the wine, under both conditions, although the differences were not significant. The results presented in Table 3.7 show that the concentration required to decrease glucose transport by 50% was lower for the sample subjected to the gastrointestinal digestion but both samples were equally more active in sodium-dependent conditions (lower IC_{50}). The inhibitory potential of apple and strawberry extracts has been reported in the literature. Under sodium-dependent and sodium-free conditions, both Aveleda Alvarinho (2014) and its digested fraction were more potent inhibitors of glucose transport than the strawberry extract. On the other hand, apple extract and wine yielded similar IC_{50} values under sodium-dependent conditions. However, when sodium was absent from the medium, apple extract demonstrated to be a more potent inhibitor of GLUT2 than Aveleda Alvarinho (2014) [73].

The effect of white wine and gastrointestinal digested white wine on glucose uptake by Caco-2 cells was also evaluated, under sodium-dependent and sodium-free conditions. In Figure 3.15 the glucose cellular uptake under sodium-dependent (Figure 3.15A) and sodium-free (Figure 3.15B) conditions of Aveleda Alvarinho (2014) and its gastrointestinal digested fraction is presented.

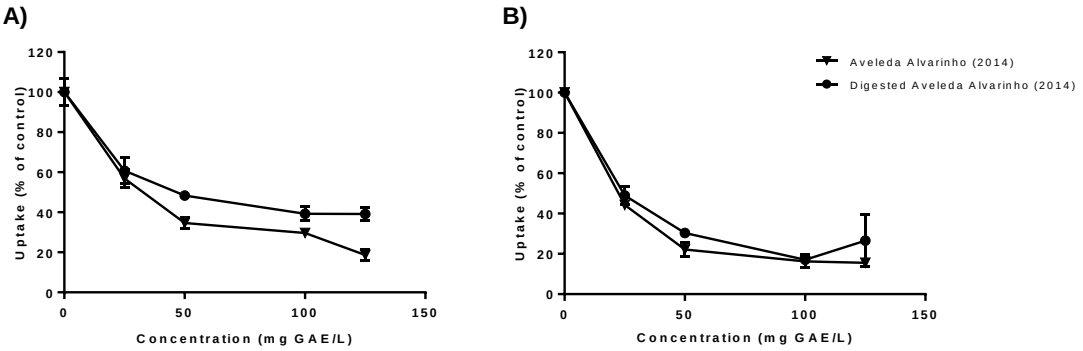

Figure 3.15: Effect of Aveleda Alvarinho (2014) and its gastrointestinal digested fraction on glucose uptake by Caco-2 cells. Intracellular concentration of glucose was measured under sodium-dependent (A) and sodium-free (B) conditions. Results are expressed as mean ± SD of two independent experiments.

The results presented in Figure 3.15 suggest that both samples were more effective under sodium-free conditions. In the case of sodium-dependent conditions, polyphenols of Aveleda Alvarinho (2014) revealed to be more efficient inhibitors of glucose uptake than its digested fraction. Because the determined uptake percentages barely exceeded 50% inhibition, lower concentrations should be tested in order to determine IC_{50} values. Overall, both samples were effective inhibitors of glucose uptake under sodium-dependent and sodium-free conditions.

Although the concentrations of phenolic compounds present in the wine decreased after gastric digestion, an increase in phenolic content was observed after intestinal digestion and this intestinal digested fraction reduced glucose transport more efficiently. Phenolic compounds are subject to metabolism in the small intestine, which results in structural changes that could result in altered biological activity [133,134]. In fact, it has been reported that metabolites from dietary phenolic compounds can be more active than their precursors in inhibiting glucose transport across Caco-2 cell monolayers [132]. Since the required amount of both samples to decrease glucose transport is lower in sodium-dependent conditions and no significant differences were found in uptake between both conditions, this suggests that SGLT1 could be the component of glucose transport in the small intestine more sensitive to the action of white wine phenolic compounds. Phenolic compounds from other plant-derived beverages have shown similar glucose transport inhibition mechanism. In particular, green tea galloylated polyphenols have been reported as inhibitors of glucose transport through SGLT1 competitive inhibition, in brush border membrane vesicles from rabbit small intestine [135].

Aiming at identifying bioactive compounds derived from white wine that could exhibit inhibitory effect in glucose transport, standard phenolic compounds, namely *p*-coumaric acid, gallic acid, resveratrol and epicatechin, were selected. Gallic and *p*-coumaric acids were abundant phenolic acids present in Aveleda Alvarinho. Epicatechin was also present at high concentration in the wine and was reported in the literature as a potential glucose transport inhibitor [135]. Although resveratrol was not present in this white wine, it is a very promising compound having been already reported as a potential inhibitor of SGLT1 glucose transporter [77]. These compounds were tested at 2.5 mM concentration and preliminary results of their effect on glucose transport, under both sodium-dependent and sodium-free conditions, are presented in Figures 3.16.

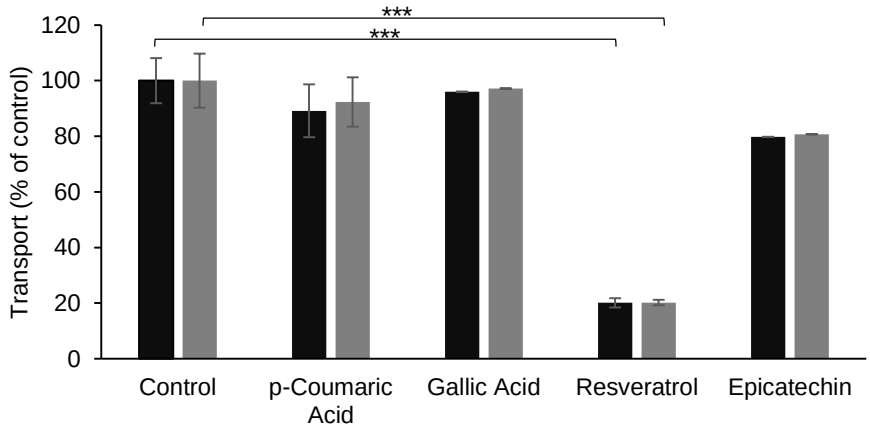

Figure 3.16: Effect on glucose transport across Caco-2 cells monolayers of *p*-coumaric acid, gallic acid, resveratrol and epicatechin at 2.5 mM concentration. Concentration of transported glucose was measured under sodium-dependent (black) and sodium-free (grey) conditions. Results are expressed as mean ± SD of duplicate. Significant differences from the control are expressed with asterisks (ns $P > 0.05$ and ***$P < 0.001$) by one-way ANOVA.

Results from Figure 3.16 (2.5 mM concentration) showed that the pure phenolic acids did not significantly affect glucose transport. In contrast, resveratrol exerted a significant ($P < 0.001$) decrease in basolateral glucose concentration, inhibiting glucose transport by approximately 80%. Epicatechin led to a more modest inhibition of approximately 20%, however this effect was not significant ($P > 0.05$). No major differences were observed between sodium-dependent and sodium-free conditions.

The most promising compounds were analyzed at a lower concentration (0.5 mM), represented in Figure 3.17.

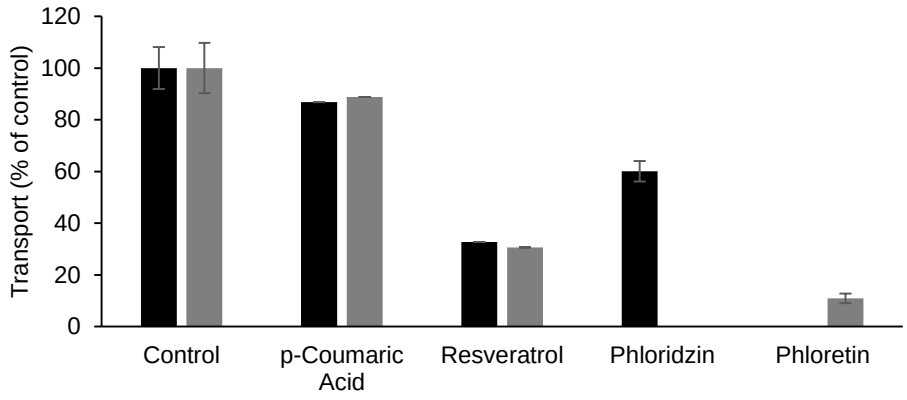

Figure 3.17: Effect on glucose transport across Caco-2 cells monolayers of *p*-coumaric acid, resveratrol, phloridzin and phloretin at 0.5 mM concentration. Concentration of transported glucose was measured under sodium-dependent (black) and sodium-free (grey) conditions. Results are expressed as mean ± SD of duplicate.

The results presented in Figure 3.17 showed that resveratrol and *p*-coumaric acid in both conditions did not show a different effect from that observed at 2.5 mM (Figure 3.16), suggesting that the inhibition was not dose-dependent. Resveratrol revealed to be a more potent inhibitor of glucose transport than phloridzin, under sodium-dependent conditions. However, under sodium-free conditions, phloretin displayed the most effective inhibition. More studies should be performed in order to confirm these results, as well as to assess the dependence of epicatechin effect with concentration.

Overall, the obtained results for pure compounds were in line with the existing studies in the literature. Most phenolic acids were reported as weak inhibitors of glucose uptake, *in vitro*. Gallic and caffeic acids reduced glucose uptake by rat brush border membrane vesicles, under sodium-dependent conditions, by 10% and 35% [60]. In Caco-2 cells, both compounds, as well as other phenolic acids, did not show significant effects on glucose uptake [61]. However*, in vivo* studies with diabetic rats demonstrated that intravenous administration of caffeic acid and oral administration of an extract containing high concentration of chlorogenic acid decreased plasma glucose concentration, suggesting that phenolic acids could have an effect as anti-hyperglycemic agents [136,137]. The effect of flavonoids on intestinal glucose transport by GLUT2 expressed in *Xenopus laevis* oocytes has been described [65]. In contrast with the results presented in Figures 3.16 and 3.17, epichatechin was reported as having a significant inhibitory effect on glucose uptake by Caco-2 cells under sodium-dependent conditions [61]. Since epicatechin was quantified in Aveleda Alvarinho (2014) in a relatively high concentration, further studies should be done to evaluate the anti-hyperglycemic potential of this phenolic compound. The potential benefits of resveratrol in several diseases are well documented in the literature, although its potential to modulate glucose absorption was not much studied. However, it was demonstrated that this phenolic compound could decrease glucose transport and uptake in the intestine by inhibiting SGLT1 in porcine jejunum and ileum [77]. Although resveratrol was not identified in the tested white wine it is an important constituent of grapes and by-products, being found in red wine and some white wines [105]. Thus, resveratrol extracted from winemaking by-products could be incorporated in white wine without dramatically change its chemical and sensorial proprieties. Therefore, there is interest to explore and study the anti-hyperglycemic potential of resveratrol, as well as the extent of how gastrointestinal digestion affects its stability and what transformations could occur before the compound reaches the target.

Literature reports on phenol-rich all-extracts and beverages demonstrate that the mixtures usually produced more significant effects on glucose transport and uptake than the pure identified phenolic compounds [132,138]. The results presented above evidence the same: white wine and digested wine produced a more potent effect than the pure compounds identified in the samples. These observations could be explained by the fact that, in a mixture, phenols can interact with one another, acting additively or synergistically, leading to enhancement of their activity [139,140].

4. Conclusions

Although the beneficial effect of moderate red wine consumption in Health is well known and widely supported by scientific evidence, there are few reports evaluating the bioactivity of white wines probably due to their lower content in phenolic compounds. In this thesis, white wines derived from Douro and Alentejo, produced with different grape varieties, were investigated for their content in phenolic compounds and their capacity in inhibiting carbohydrate-digestion enzymes and intestinal glucose transporters, aiming at identifying promising inhibitory compounds with positive impact in type 2 diabetes.

Among the four white wines analyzed, Aveleda Alvarinho distinguished itself for presenting a higher phenolic content, particularly in phenolic acids such as gallic, caffeic and *p*-coumaric acid. Despite the fact that the inhibitory effect of this wine against α-glucosidase activity was not relevant when compared with other plant-derived products (e.g. teas, wine pomace and berry extracts), the phenolic fraction of this wine enhances the inhibitory potential of acarbose, a commonly used anti-diabetic drug that impairs glucose absorption in the small intestine through inhibition of these carbohydrate-digestion enzymes. This effect was suggested to be related with the phytochemical content of this wine, gallic acid being pointed as the main contributor for this effect. Importantly, Aveleda Alvarinho demonstrated inhibitory capacity of glucose transport, acting on SGLT1 and GLUT2 intestinal glucose transporters in Caco-2 cell line and the *in vitro* gastrointestinal digestion process did not compromise the bioactivity of the wine. Amongst the pure phenolic compounds tested, resveratrol emerged as a potential inhibitor of both glucose transporters, although the inhibitory potential of other phenolic compounds should also be explored.

Overall, white wine constitutes a promising source of bioactive compounds with positive impact in T2DM. Although the concentration of bioactive compounds in white wine is not very high, strategies can be adopted to enhance the health promoting effect, such as fortifying the wine with bioactive compounds. Future studies should be performed to identify more bioactive compounds from white wine, such as ε-viniferin and *trans*-piceid, as well a full characterization of the gastric and intestinal fractions, in order to evaluate the structural biotransformations that phenolic compounds undergo until reaching their sites of action. Moreover, the study of phenolic compounds involved in the inhibition of glucose transport is required to understand the mechanism of action of white wine, allowing to explore these effects in terms of functional food.

5. References

[1] WHO, Global Health Risks: Mortality and burden of disease attributable to selected major risks, Bull. World Health Organ. 87 (2009) 646–646. doi:10.2471/BLT.09.070565.

[2] S. Edition, Diabetes, 2015. doi:10.1289/image.ehp.v119.i03.

[3] OND, Diabetes: Factos e Números do Ano de 2014, 2015.

[4] O.H. Data, Causas de morte Causas de morte 2013, (2015) 1–8.

[5] American Diabetes Association, Diagnosis and classification of diabetes mellitus, Diabetes Care. 33 (2010). doi:10.2337/dc10-S062.

[6] M.B. Schulze, F.B. Hu, PRIMARY PREVENTION OF DIABETES: What Can Be Done and How Much Can Be Prevented, Annu. Rev. Public Health. 26 (2004) 445–467. doi:10.1146/annurev.publhealth.26.021304.144532.

[7] P. Hossain, B. Kawar, M. El Nahas, Obesity and diabetes in the developing world: a growing challenge, N. Engl. J. Med. 356 (2007) 213–215. doi:10.1056/NEJMp068177.

[8] R.H. Eckel, S.M. Grundy, P.Z. Zimmet, The metabolic syndrome, Lancet. 365 (2005) 1415–1428. doi:10.1016/S0140-6736(05)66378-7.

[9] B. Gallwitz, Implications of postprandial glucose and weight control in people with type 2 diabetes: understanding and implementing the International Diabetes Federation guidelines., Diabetes Care. 32 Suppl 2 (2009) 0–3. doi:10.2337/dc09-S331.

[10] E. Bonora, Postprandial peaks as a risk factor for cardiovascular disease: epidemiological perspectives., Int. J. Clin. Pract. Suppl. (2002) 5–11.

[11] J.E. Shaw, A.M. Hodge, M. De Courten, P. Chitson, P.Z. Zimmet, Isolated post-challenge hyperglycaemia confirmed as a risk factor for mortality, Diabetologia. 42 (1999) 1050–1054. doi:10.1007/s001250051269.

[12] A. Ceriello, J. Davidson, M. Hanefeld, L. Leiter, L. Monnier, D. Owens, N. Tajima, J. Tuomilehto, Postprandial hyperglycaemia and cardiovascular complications of diabetes: An update, Nutr. Metab. Cardiovasc. Dis. 16 (2006) 453–456. doi:10.1016/j.numecd.2006.05.006.

[13] S.E. Inzucchi, R.M. Bergenstal, J.B. Buse, M. Diamant, E. Ferrannini, M. Nauck, A.L. Peters, A. Tsapas, R. Wender, D.R. Matthews, Management of Hyperglycemia in Type 2 Diabetes, 2015: A Patient-Centered Approach: Update to a position statement of the american diabetes association and the european association for the study of diabetes, Diabetes Care. 38 (2015) 140–149. doi:10.2337/dc14-2441.

[14] L. Lin, S.W. Yee, R.B. Kim, K.M. Giacomini, SLC transporters as therapeutic targets : emerging opportunities, Nat. Publ. Gr. 14 (2015) 543–560. doi:10.1038/nrd4626.

[15] G.L. Kellett, E. Brot-Laroche, Apical GLUT2: A major pathway of intestinal sugar absorption, Diabetes. 54 (2005) 3056–3062. doi:10.2337/diabetes.54.10.3056.

[16] E.H. Van Beers, A. Hans, R.J. Grand, W.C. Alexandra, Intestinal Brush Border Glycohydrolases : Structure , Function , and Development, 30 (1995) 197–262.

[17] C. Castaneda-Sceppa, F. Castaneda, Sodium-dependent glucose transporter protein as a potential therapeutic target for improving glycemic control in diabetes, Nutr. Rev. 69 (2011) 720–729. doi:10.1111/j.1753-4887.2011.00423.x.

[18] D.C. Whitcomb, M.E. Lowe, Human pancreatic digestive enzymes., Dig. Dis. Sci. 52 (2007) 1–17. doi:10.1007/s10620-006-9589-z.

[19] F. Payan, Structural basis for the inhibition of mammalian and insect α-amylases by plant protein inhibitors, Biochim. Biophys. Acta - Proteins Proteomics. 1696 (2004) 171–180. doi:10.1016/j.bbapap.2003.10.012.

[20] M. Okuyama, W. Saburi, H. Mori, A. Kimura, α-Glucosidases and α-1,4-glucan lyases: structures, functions, and physiological actions, Cell. Mol. Life Sci. (2016). doi:10.1007/s00018-016-2247-5.

[21] S. Chiba, Molecular mechanism in alpha-glucosidase and glucoamylase., Biosci. Biotechnol. Biochem. 61 (1997) 1233–1239. doi:10.1271/bbb.61.1233.

[22] A.J. Scheen, Is there a role for alpha-glucosidase inhibitors in the prevention of type 2 diabetes mellitus, Drugs. 63 (2003) 933–951.

[23] P.J. Lefebvre, A.J. Scheen, Management of non-insulin-dependent diabetes mellitus., Drugs. 44 Suppl 3 (1992) 29–38.

[24] H. Bischoff, The mechanism of alpha-glucosidase inhibition in the management of diabetes., Clin. Invest. Med. 18 (1995) 303–11.

[25] J. Hoffmann, M. Spengler, Efficacy of 24-week monotherapy with acarbose, metformin, or placebo in dietary-treated NIDDM patients: The essen-II study, Am. J. Med. 103 (1997)

483–490. doi:10.1016/S0002-9343(97)00252-0.
[26] J.-L. Chiasson, R.G. Josse, R. Gomis, M. Hanefeld, A. Karasik, M. Laakso, Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial, Lancet. 359 (2002) 2072–2077. doi:10.1016/S0140-6736(02)08905-5.
[27] J.T. Lettieri, B. Dain, Effects of beano on the tolerability and pharmacodynamics of acarbose, Clin. Ther. 20 (1998) 497–504. doi:10.1016/S0149-2918(98)80059-3.
[28] Y.-I.I. Kwon, D.A. Vattem, K. Shetty, Evaluation of clonal herbs of Lamiaceae species for management of diabetes and hypertension, Asia Pac. J. Clin. Nutr. 15 (2006) 107–118. doi:10.1210/er.19.5.583.
[29] Z. Yin, W. Zhang, F. Feng, Y. Zhang, W. Kang, α-Glucosidase inhibitors isolated from medicinal plants, Food Sci. Hum. Wellness. 3 (2014) 136–174. doi:10.1016/j.fshw.2014.11.003.
[30] A. Scheepers, H.-G. Joost, A. Schürmann, The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function., J. Parenter. Enter. Nutr. 28 (2004) 364–371. doi:10.1177/0148607104028005364.
[31] G.L. Kellett, P.A. Helliwell, The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane, Biochem. Soc. 162 (2000) 155–162.
[32] E.M. Wright, B.A. Hirayama, D.F. Loo, Active sugar transport in health and disease, in: J. Intern. Med., 2007: pp. 32–43. doi:10.1111/j.1365-2796.2006.01746.x.
[33] Nakazawa F., The influence of phlorhizin on intestinal absorption., Tohoku J. Exp. Med. 3 (1922) 288–295.
[34] F. Alvarado, Hypothesis for the interaction of phlorizin and phloretin with membrane carriers for sugars., Biochim. Biophys. Acta. 135 (1967) 483–495. doi:10.1016/0005-2736(67)90038-7.
[35] Donhoffer S., Uber die elektive resorption der zucker, Arch. Exp. Pathol. Pharmakol. 177 (1935) 689–92.
[36] J.L. Madara, J.R. Pappenheimer, Structural basis for physiological regulation of paracellular pathways in intestinal epithelia., J. Membr. Biol. 100 (1987) 149–64.
[37] G.L. Kellett, E. Brot-Laroche, O.J. Mace, A. Leturque, Sugar absorption in the intestine: the role of GLUT2., Annu. Rev. Nutr. 28 (2008) 35–54. doi:10.1146/annurev.nutr.28.061807.155518.
[38] G.L. Kellett, E. Brot-Laroche, Apical GLUT2: A major pathway of intestinal sugar absorption, Diabetes. 54 (2005) 3056–3062. doi:10.2337/diabetes.54.10.3056.
[39] G.M. Cragg, D.J. Newman, Natural products: A continuing source of novel drug leads, Biochim. Biophys. Acta - Gen. Subj. 1830 (2013) 3670–3695. doi:10.1016/j.bbagen.2013.02.008.
[40] A.L. Harvey, Natural products in drug discovery, Drug Discov. Today. 13 (2008) 894–901. doi:10.1016/j.drudis.2008.07.004.
[41] M.S. Butler, A.A.B. Robertson, M.A. Cooper, Natural product and natural product derived drugs in clinical trials, Nat. Prod. Rep. 31 (2014) 1612–1661. doi:10.1039/c4np00064a.
[42] V. Brower, Nutraceuticals: poised for a healthy slice of the healthcare market?, Nat. Biotechnol. 16 (1998) 728–31. doi:10.1038/nbt0898-728.
[43] J.C. Espín, M.T. García-Conesa, F.A. Tomás-Barberán, Nutraceuticals: Facts and fiction, Phytochemistry. 68 (2007) 2986–3008. doi:10.1016/j.phytochem.2007.09.014.
[44] S.R.B.M. Eussen, H. Verhagen, O.H. Klungel, J. Garssen, H. Van Loveren, H.J. Van Kranen, C.J.M. Rompelberg, Functional foods and dietary supplements: Products at the interface between pharma and nutrition, in: Eur. J. Pharmacol., 2011: pp. S2-9. doi:10.1016/j.ejphar.2011.07.008.
[45] Y. Kim, J.B. Keogh, P.M. Clifton, Polyphenols and Glycemic Control., Nutrients. 8 (2016) E17. doi:10.3390/nu8010017.
[46] I. Ignat, I. Volf, V.I. Popa, A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables., Food Chem. 126 (2011) 1821–35. doi:10.1016/j.foodchem.2010.12.026.
[47] K.W.J. Wahle, I. Brown, D. Rotondo, S.D. Heys, Plant phenolics in the prevention and treatment of cancer, Adv. Exp. Med. Biol. 698 (2010) 36–51. doi:10.1007/978-1-4419-7347-4_4.
[48] Y. Kim, J.B. Keogh, P.M. Clifton, Polyphenols and Glycemic Control., Nutrients. 8 (2016) E17. doi:10.3390/nu8010017.
[49] E.M. Williamson, Synergy and other interactions in phytomedicines., Phytomedicine. 8

(2001) 401–9. doi:10.1078/0944-7113-00060.
[50] U. Lewandowska, S. Gorlach, K. Owczarek, E. Hrabec, K. Szewczyk, Synergistic interactions between anticancer chemotherapeutics and phenolic compounds and anticancer synergy between polyphenols, Postepy Hig. Med. Dosw. 68 (2014) 528–540. doi:10.5604/17322693.1102278.
[51] A.A. Alshatwi, V.S. Periasamy, J. Athinarayanan, R. Elango, Synergistic anticancer activity of dietary tea polyphenols and bleomycin hydrochloride in human cervical cancer cell: Caspase-dependent and independent apoptotic pathways, Chem. Biol. Interact. 247 (2016) 1–10. doi:10.1016/j.cbi.2016.01.012.
[52] A.S. Boath, D. Stewart, G.J. McDougall, Berry components inhibit α-glucosidase in vitro: synergies between acarbose and polyphenols from black currant and rowanberry., Food Chem. 135 (2012) 929–36. doi:10.1016/j.foodchem.2012.06.065.
[53] G. Cavallini, S. Straniero, A. Donati, E. Bergamini, Resveratrol requires red wine polyphenols for optimum antioxidant activity, J. Nutr. Heal. Aging. 20 (2015) 540–5. doi:10.1007/s12603-015-0611-z.
[54] Z. Gazova, K. Siposova, E. Kurin, P. Mučaji, M. Nagy, Amyloid aggregation of lysozyme: The synergy study of red wine polyphenols, Proteins Struct. Funct. Bioinforma. 81 (2013) 994–1004. doi:10.1002/prot.24250.
[55] G. Li, L. Ruan, R. Chen, R. Wang, X. Xie, M. Zhang, L. Chen, Q. Yan, M. Reed, J. Chen, Y. Xu, J. Pan, W. Huang, Synergistic antidepressant-like effect of ferulic acid in combination with piperine: involvement of monoaminergic system, Metab. Brain Dis. 30 (2015) 1505–1514. doi:10.1007/s11011-015-9704-y.
[56] P. Pignatelli, A. Ghiselli, B. Buchetti, R. Carnevale, F. Natella, G. German??, F. Fimognari, S. Di Santo, L. Lenti, F. Violi, Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine, Atherosclerosis. 188 (2006) 77–83. doi:10.1016/j.atherosclerosis.2005.10.025.
[57] F.F. Anhê, Y. Desjardins, G. Pilon, S. Dudonné, M.I. Genovese, F.M. Lajolo, A. Marette, Polyphenols and type 2 diabetes: A prospective review, PharmaNutrition. 1 (2013) 105–114. doi:10.1016/j.phanu.2013.07.004.
[58] M. de Bock, J.G.B. Derraik, W.S. Cutfield, Polyphenols and Glucose Homeostasis in Humans, J. Acad. Nutr. Diet. 112 (2012) 808–815. doi:10.1016/j.jand.2012.01.018.
[59] A. Ishikawa, H. Yamashita, M. Hiemori, E. Inagaki, M. Kimoto, M. Okamoto, H. Tsuji, A.N. Memon, A. Mohammadio, Y. Natori, Characterization of inhibitors of postprandial hyperglycemia from the leaves of Nerium indicum., J. Nutr. Sci. Vitaminol. (Tokyo). 53 (2007) 166–173. doi:10.3177/jnsv.53.166.
[60] C.A. Welsch, P.A. Lachance, B.P. Wasserman, Dietary phenolic compounds: inhibition of Na+-dependent D-glucose uptake in rat intestinal brush border membrane vesicles., J. Nutr. 119 (1989) 1698–1704.
[61] K. Johnston, P. Sharp, M. Clifford, L. Morgan, Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells, FEBS Lett. 579 (2005) 1653–1657. doi:10.1016/j.febslet.2004.12.099.
[62] B.K. Chakravarthy, S. Gupta, K.D. Gode, Functional beta cell regeneration in the islets of pancreas in alloxan induced diabetic rats by (-)-epicatechin, Life Sci. 31 (1982) 2693–2697. doi:10.1016/0024-3205(82)90713-5.
[63] C.S.T. Hii, S.L. Howell, Effects of flavonoids on insulin secretion and 45Ca2+ handling in rat islets of Langerhans, J. Endocrinol. 107 (1985) 1–8. doi:10.1677/joe.0.1070001.
[64] K. Tadera, Y. Minami, K. Takamatsu, T. Matsuoka, Inhibition of alpha-glucosidase and alpha-amylase by flavonoids., J. Nutr. Sci. Vitaminol. (Tokyo). 52 (2006) 149–153. doi:10.3177/jnsv.52.149.
[65] O. Kwon, P. Eck, S. Chen, C.P. Corpe, J.-H. Lee, M. Kruhlak, M. Levine, Inhibition of the intestinal glucose transporter GLUT2 by flavonoids., FASEB J. 21 (2007) 366–377. doi:10.1096/fj.06-6620com.
[66] C. Schulze, A. Bangert, B. Schwanck, H. Vollert, W. Blaschek, H. Daniel, Extracts and flavonoids from onion inhibit the intestinal sodium-coupled glucose transporter 1 (SGLT1) in vitro but show no anti-hyperglycaemic effects in vivo in normoglycaemic mice and human volunteers, J. Funct. Foods. 18 (2015) 117–128. doi:10.1016/j.jff.2015.06.037.
[67] Y. Narita, K. Inouye, Kinetic analysis and mechanism on the inhibition of chlorogenic acid and its components against porcine pancreas alpha-amylase isozymes I and II., J. Agric. Food Chem. 57 (2009) 9218–25. doi:10.1021/jf9017383.
[68] C.A. Welsch, P.A. Lachance, B.P. Wasserman, MEffects of native and oxidized phenolic

compounds on sucrase activity in rat brush border membrane vesicles, J. Nutr. 119 (1989) 1737–1740.
[69] S. Adisakwattana, P. Chantarasinlapin, H. Thammarat, S. Yibchok-Anun, A series of cinnamic acid derivatives and their inhibitory activity on intestinal alpha-glucosidase., J. Enzyme Inhib. Med. Chem. 24 (2009) 1194–1200. doi:10.1080/14756360902779326.
[70] I. Funke, M.F. Melzig, Effect of different phenolic compounds on alpha-amylase activity: screening by microplate-reader based kinetic assay., Pharmazie. 60 (2005) 796–7.
[71] K. Iwai, M.-Y. Kim, A. Onodera, H. Matsue, Alpha-glucosidase inhibitory and antihyperglycemic effects of polyphenols in the fruit of Viburnum dilatatum Thunb., J. Agric. Food Chem. 54 (2006) 4588–92. doi:10.1021/jf0606353.
[72] T. Wang, X. Li, B. Zhou, H. Li, J. Zeng, W. Gao, Anti-diabetic activity in type 2 diabetic mice and α-glucosidase inhibitory, antioxidant and anti-inflammatory potential of chemically profiled pear peel and pulp extracts (Pyrus spp.), J. Funct. Foods. 13 (2015) 276–288. doi:10.1016/j.jff.2014.12.049.
[73] S. Manzano, G. Williamson, Polyphenols and phenolic acids from strawberry and apple decrease glucose uptake and transport by human intestinal Caco-2 cells., Mol. Nutr. Food Res. 54 (2010) 1773–1780. doi:10.1002/mnfr.201000019.
[74] S. Adisakwattana, P. Moonsan, S. Yibchok-Anun, Insulin-releasing properties of a series of cinnamic acid derivatives in vitro and in vivo., J. Agric. Food Chem. 56 (2008) 7838–44. doi:10.1021/jf801208t.
[75] H.J. Eun, R.K. Sung, K.H. In, Y.H. Tae, Hypoglycemic effects of a phenolic acid fraction of rice bran and ferulic acid in C57BL/KsJ-db/db mice, J. Agric. Food Chem. 55 (2007) 9800–9804. doi:10.1021/jf0714463.
[76] Z. Kerem, I. Bilkis, M.A. Flaishman, L. Sivan, Antioxidant activity and inhibition of alpha-glucosidase by trans-resveratrol, piceid, and a novel trans-stilbene from the roots of Israeli Rumex bucephalophorus L., J. Agric. Food Chem. 54 (2006) 1243–1247. doi:10.1021/jf052436+.
[77] M. Guschlbauer, S. Klinger, M. Burmester, J. Horn, S.E. Kulling, G. Breves, trans-Resveratrol and ε-viniferin decrease glucose absorption in porcine jejunum and ileum in vitro., Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 165 (2013) 313–8. doi:10.1016/j.cbpa.2013.03.040.
[78] J. Pérez-Jiménez, V. Neveu, F. Vos, A. Scalbert, Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database., Eur. J. Clin. Nutr. 64 Suppl 3 (2010) S112–S120. doi:10.1038/ejcn.2010.221.
[79] A. Artero, A. Artero, J.J. Tarín, A. Cano, The impact of moderate wine consumption on health, Maturitas. 80 (2015) 3–13. doi:10.1016/j.maturitas.2014.09.007.
[80] V.G. Athyros, E.N. Liberopoulos, D.P. Mikhailidis, A.A. Papageorgiou, E.S. Ganotakis, K. Tziomalos, A.I. Kakafika, A. Karagiannis, S. Lambropoulos, M. Elisaf, Association of drinking pattern and alcohol beverage type with the prevalence of metabolic syndrome, diabetes, coronary heart disease, stroke, and peripheral arterial disease in a Mediterranean cohort., Angiology. 58 (2008) 689–97. doi:10.1177/0003319707306146.
[81] M. Boban, C. Stockley, P.-L. Teissedre, P. Restani, U. Fradera, C. Stein-Hammer, J.-C. Ruf, Drinking pattern of wine and effects on human health: why should we drink moderately and with meals?, Food Funct. 7 (2016) 2937–2942. doi:10.1039/C6FO00218H.
[82] J. Garrido, F. Borges, Wine and grape polyphenols - A chemical perspective, Food Res. Int. 54 (2013) 1844–1858. doi:10.1016/j.foodres.2013.08.002.
[83] A.L. Waterhouse, Wine phenolics., Ann. N. Y. Acad. Sci. 957 (2002) 21–36. doi:10.1111/j.1749-6632.2002.tb02903.x.
[84] A. Teixeira, J. Eiras-Dias, S.D. Castellarin, H. Gerós, Berry phenolics of grapevine under challenging environments, Int. J. Mol. Sci. 14 (2013) 18711–18739. doi:10.3390/ijms140918711.
[85] J.A. Baur, D.A. Sinclair, Therapeutic potential of resveratrol: the in vivo evidence, Nat. Rev. Drug Discov. 5 (2006) 493–506. doi:10.1038/nrd2060.
[86] R. Nakata, H. Inoue, Resveratrol and Cardiovascular Disease, Curr. Nutr. Rep. 8 (2014) 163–169. doi:10.1007/s13668-014-0084-4.
[87] L. Xiang, L. Xiao, Y. Wang, H. Li, Z. Huang, X. He, Health benefits of wine: Don't expect resveratrol too much, Food Chem. 156 (2014) 258–263. doi:10.1016/j.foodchem.2014.01.006.
[88] A.E. Bantle, W. Thomas, J.P. Bantle, Metabolic effects of alcohol in the form of wine in

persons with type 2 diabetes mellitus, Metabolism. 57 (2008) 241–245. doi:10.1016/j.metabol.2007.09.007.
[89] Y. Gepner, R. Golan, I. Harman-Boehm, Y. Henkin, D. Schwarzfuchs, I. Shelef, R. Durst, J. Kovsan, A. Bolotin, E. Leitersdorf, S. Shpitzen, S. Balag, E. Shemesh, S. Witkow, O. Tangi-Rosental, Y. Chassidim, I.F. Liberty, B. Sarusi, S. Ben-Avraham, A. Helander, U. Ceglarek, M. Stumvoll, M. Blüher, J. Thiery, A. Rudich, M.J. Stampfer, I. Shai, Effects of initiating moderate alcohol intake on cardiometabolic risk in adults with type 2 diabetes: A 2-year randomized, controlled trial, Ann. Intern. Med. 163 (2015) 569–579. doi:10.7326/M14-1650.
[90] A.M. Hodge, D.R. English, K. O'Dea, G.G. Giles, Alcohol intake, consumption pattern and beverage type, and the risk of Type 2 diabetes, Diabet. Med. 23 (2006) 690–697. doi:10.1111/j.1464-5491.2006.01864.x.
[91] A. Lavy, B. Fuhrman, A. Markel, G. Dankner, A. Ben-Amotz, D. Presser, M. Aviram, Effect of dietary supplementation of red or white wine on human blood chemistry, hematology and coagulation: favorable effect of red wine on plasma high-density lipoprotein, Ann Nutr Metab. 38 (1994) 287–294.
[92] S. Hogan, L. Zhang, J. Li, S. Sun, C. Canning, K. Zhou, Antioxidant rich grape pomace extract suppresses postprandial hyperglycemia in diabetic mice by specifically inhibiting alpha-glucosidase., Nutr. Metab. (Lond). 7 (2010) 71. doi:10.1186/1743-7075-7-71.
[93] V. Lavelli, P.S.C. Sri Harsha, P. Ferranti, A. Scarafoni, S. Iametti, Grape skin phenolics as inhibitors of mammalian α-glucosidase and α-amylase – effect of food matrix and processing on efficacy, Food Funct. 7 (2016) 1655–1663. doi:10.1039/C6FO00073H.
[94] Y.I. Kwon, E. Apostolidis, K. Shetty, Inhibitory potential of wine and tea against alpha-amylase and alpha-glucosidase for management of hyperglycemia linked to type 2 diabetes, J. Food Biochem. 32 (2008) 15–31. doi:10.1111/j.1745-4514.2007.00165.x.
[95] V.L. Singleton, J.A. Rossi, Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents, Am. J. Enol. Vitic. 16 (1965) 144–158.
[96] A.T. Serra, J. Rocha, B. Sepodes, A.A. Matias, R.P. Feliciano, A. De Carvalho, M.R. Bronze, C.M.M. Duarte, M.E. Figueira, Evaluation of cardiovascular protective effect of different apple varieties - Correlation of response with composition, Food Chem. 135 (2012) 2378–2386. doi:10.1016/j.foodchem.2012.07.067.
[97] M.M. Al-Dabbas, K. Kitahara, T. Suganuma, F. Hashimoto, K. Tadera, Antioxidant and alpha-amylase inhibitory compounds from aerial parts of Varthemia iphionoides Boiss., Biosci. Biotechnol. Biochem. 70 (2006) 2178–2184. doi:10.1271/bbb.60132.
[98] T.T. Mai, N. Van Chuyen, Anti-Hyperglycemic Activity of an Aqueous Extract from Flower Buds of *Cleistocalyx operculatus (Roxb.) Merr and Perry*, Biosci. Biotechnol. Biochem. 71 (2007) 69–76. doi:10.1271/bbb.60373.
[99] T.C. Chou, P. Talalay, Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, Adv. Enzyme Regul. 22 (1984) 27–55. doi:10.1016/0065-2571(84)90007-4.
[100] M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrì, R. Boutrou, F.M. Corredig, D. Dupont, F.C. Dufour, L. Egger, M. Golding, L.S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D.J. Mcclements, O. Enard, I. Recio, C.N. Santos, R.P. Singh, G.E. Vegarud, M.S.J. Wickham, W. Weitschies, A. Brodkorb, A standardised static in vitro digestion method suitable for food – an international consensus, Food Funct. Food Funct. 5 (2014) 1113–1124. doi:10.1039/c3fo60702j.
[101] Y. Sambuy, I. De Angelis, G. Ranaldi, M.L. Scarino, A. Stammati, F. Zucco, The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics, Cell Biol. Toxicol. 21 (2005) 1–26. doi:10.1007/s10565-005-0085-6.
[102] A.T. Serra, A.A. Matias, A.P.C. Almeida, M.R. Bronze, P.M. Alves, H.C. De Sousa, C.M.M. Duarte, Processing cherries (Prunus avium) using supercritical fluid technology. Part 2. Evaluation of SCF extracts as promising natural chemotherapeutical agents, J. Supercrit. Fluids. 55 (2011) 1007–1013. doi:10.1016/j.supflu.2010.06.006.
[103] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254. doi:10.1016/0003-2697(76)90527-3.
[104] C.M.M. Duarte, A.T. Serra, A.A. Matias, M. do R. Bronze, Vinho Branco - uma opção saudável com impacto na diabetes, 2013.

[105] C. Tourtoglou, N. Nenadis, A. Paraskevopoulou, Phenolic composition and radical scavenging activity of commercial Greek white wines from Vitis vinifera L. cv. Malagousia, J. Food Compos. Anal. 33 (2014) 166–174. doi:10.1016/j.jfca.2013.12.009.
[106] J. Burns, P.T. Gardner, J. O'Neil, S. Crawford, I. Morecroft, D.B. McPhail, C. Lister, D. Matthews, M.R. MacLean, M.E.J. Lean, G.G. Duthie, A. Crozier, Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines, J. Agric. Food Chem. 48 (2000) 220–230. doi:10.1021/jf9909757.
[107] Y.-I. Kwon, H.-J. Son, K.S. Moon, J.K. Kim, J.-G. Kim, H.-S. Chun, S.K. Ahn, C. Il Hong, Novel alpha-glucosidase inhibitors, CKD-711 and CKD-711a produced by Streptomyces sp. CK-4416. II. Biological properties., J. Antibiot. (Tokyo). 55 (2002) 462–6.
[108] D.E. La, K.E.Y. Words, Spectrophotometric Methods for the Analysis of Polysaccharide Levels in Winemaking Products, Am. J. Enol. Vitic. 46 (1995) 564–570.
[109] A. Bellesia, D. Tagliazucchi, Cocoa brew inhibits in vitro alpha-glucosidase activity: The role of polyphenols and high molecular weight compounds, Food Res. Int. 63 (2014) 439–445. doi:10.1016/j.foodres.2014.03.047.
[110] T. Satoh, M. Igarashi, S. Yamada, N. Takahashi, K. Watanabe, Inhibitory effect of black tea and its combination with acarbose on small intestinal α-glucosidase activity, J. Ethnopharmacol. 161 (2015) 147–155. doi:10.1016/j.jep.2014.12.009.
[111] O. Kamiyama, F. Sanae, K. Ikeda, Y. Higashi, Y. Minami, N. Asano, I. Adachi, A. Kato, In vitro inhibition of α-glucosidases and glycogen phosphorylase by catechin gallates in green tea, Food Chem. 122 (2010) 1061–1066. doi:10.1016/j.foodchem.2010.03.075.
[112] J. Zhao, X.-W. Zhou, X.-B. Chen, Q.-X. Wang, α-glucosidase inhibitory constituents from Toona sinensis, Chem. Nat. Compd. 45 (2009) 244–246. doi:10.1007/s10600-009-9289-y.
[113] T.-C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies., Pharmacol. Rev. 58 (2006) 621–81. doi:10.1124/pr.58.3.10.
[114] V. Katalinić, M. Milos, D. Modun, I. Musić, M. Boban, Antioxidant effectiveness of selected wines in comparison with (+)-catechin, Food Chem. 86 (2004) 593–600. doi:10.1016/j.foodchem.2003.10.007.
[115] M.T. Ribeiro De Lima, P. Waffo-Téguo, P.L. Teissedre, A. Pujolas, J. Vercauteren, J.C. Cabanis, J.M. Mérillon, Determination of stilbenes (trans-astringin, cis- and trans-piceid, and cis- and trans-resveratrol) in Portuguese wines, J. Agric. Food Chem. 47 (1999) 2666–2670. doi:10.1021/jf9900884.
[116] R.R. Tian, Q.H. Pan, J.C. Zhan, J.M. Li, S.B. Wan, Q.H. Zhang, W.D. Huang, Comparison of phenolic acids and flavan-3-ols during wine fermentation of grapes with different harvest times, Molecules. 14 (2009) 827–838. doi:10.3390/molecules14020827.
[117] P. Jandera, V. Skeifíková, L. Rehová, T. Hájek, L. Baldriánová, G. Skopová, V. Kellner, A. Horna, RP-HPLC analysis of phenolic compounds and flavonoids in beverages and plant extracts using a CoulArray detector., J. Sep. Sci. 28 (2005) 1005–22.
[118] X. Vitrac, J.P. Monti, J. Vercauteren, G. Deffieux, J.M. Mérillon, Direct liquid chromatographic analysis of resveratrol derivatives and flavanonols in wines with absorbance and fluorescence detection, in: Anal. Chim. Acta, 2002: pp. 103–110. doi:10.1016/S0003-2670(01)01498-2.
[119] E.N. Frankel, A.L. Waterhouse, P.L. Teissedrespt, Principal phenolic phytochemicals in selected california wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins, J. Agric. Food Chem. 43 (1995) 890–894. doi:10.1021/jf00052a008.
[120] P. Li, X.Q. Wang, H.Z. Wang, Y.N. Wu, High performance liquid chromatographic determination of phenolic acids in fruits and vegetables., Biomed. Environ. Sci. 6 (1993) 389–98.
[121] D. Tagliazucchi, E. Verzelloni, D. Bertolini, A. Conte, In vitro bio-accessibility and antioxidant activity of grape polyphenols, Food Chem. 120 (2010) 599–606. doi:10.1016/j.foodchem.2009.10.030.
[122] A. Cilla, S. Perales, M.J. Lagarda, R. Barberá, G. Clemente, R. Farré, Influence of storage and in vitro gastrointestinal digestion on total antioxidant capacity of fruit beverages, J. Food Compos. Anal. 24 (2011) 87–94. doi:10.1016/j.jfca.2010.03.029.
[123] V.L. Singleton, J.A. Rossi Jr, Colorimetry of total phenolics with phosphomolybdic-phosphothungstic acid reagents, Am. J. Enol. Vitic. 16 (1965) 144–158. doi:citeulike-article-id:7170825.
[124] M. V. Martínez-Ortega, M.C. García-Parrilla, A.M. Troncoso, Changes in phenolic

composition of wines submitted to in vitro dissolution tests, Food Chem. 73 (2001) 11–16. doi:10.1016/S0308-8146(00)00270-3.
[125] H. Sakakibara, Y. Honda, S. Nakagawa, H. Ashida, K. Kanazawa, Simultaneous determination of all polyphenols in vegetables, fruits, and teas, J. Agric. Food Chem. 51 (2003) 571–581. doi:10.1021/jf020926l.
[126] M. Gumienna, M. Lasik, Z. Czarnecki, Bioconversion of grape and chokeberry wine polyphenols during simulated gastrointestinal in vitro digestion, Int. J. Food Sci. Nutr. 62 (2011) 226–233. doi:10.3109/09637486.2010.532115.
[127] J.M. Ricardo da Silva, J. Rigaud, V. Cheynier, A. Cheminat, M. Moutounet, Procyanidin dimers and trimers from grape seeds, Phytochemistry. 30 (1991) 1259–1264. doi:10.1016/S0031-9422(00)95213-0.
[128] Q.Y. Zhu, R.R. Holt, S.A. Lazarus, J.L. Ensunsa, J.F. Hammerstone, H.H. Schmitz, C.L. Keen, Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa, J. Agric. Food Chem. 50 (2002) 1700–1705. doi:10.1021/jf011228o.
[129] I.R. Record, J.M. Lane, Simulated intestinal digestion of green and black teas, Food Chem. 73 (2001) 481–486. doi:10.1016/S0308-8146(01)00131-5.
[130] M.-J. Bermúdez-Soto, F.-A. Tomás-Barberán, M.-T. García-Conesa, Stability of polyphenols in chokeberry (Aronia melanocarpa) subjected to in vitro gastric and pancreatic digestion, Food Chem. 102 (2007) 865–874. doi:10.1016/j.foodchem.2006.06.025.
[131] L. Mahraoui, A. Rodolosse, A. Barbat, E. Dussaulx, A. Zweibaum, M. Rousset, E. Brot-Laroche, Presence and differential expression of SGLT1, GLUT1, GLUT2, GLUT3 and GLUT5 hexose-transporter mRNAs in Caco-2 cell clones in relation to cell growth and glucose consumption., Biochem. J. 298 Pt 3 (1994) 629–33.
[132] T.L. Farrell, S.L. Ellam, T. Forrelli, G. Williamson, Attenuation of glucose transport across Caco-2 cell monolayers by a polyphenol-rich herbal extract: Interactions with SGLT1 and GLUT2 transporters, BioFactors. 39 (2013) 448–456. doi:10.1002/biof.1090.
[133] J. Bouayed, H. Deußer, L. Hoffmann, T. Bohn, Bioaccessible and dialysable polyphenols in selected apple varieties following in vitro digestion vs. their native patterns, Food Chem. 131 (2012) 1466–1472. doi:10.1016/j.foodchem.2011.10.030.
[134] R. Pinacho, R.Y. Cavero, I. Astiasarán, D. Ansorena, M.I. Calvo, Phenolic compounds of blackthorn (Prunus spinosa L.) and influence of in vitro digestion on their antioxidant capacity, J. Funct. Foods. 19 (2015) 49–62. doi:10.1016/j.jff.2015.09.015.
[135] Y. Kobayashi, M. Suzuki, H. Satsu, S. Arai, Y. Hara, K. Suzuki, Y. Miyamoto, M. Shimizu, Green tea polyphenols inhibit the sodium-dependent glucose transporter of intestinal epithelial cells by a competitive mechanism, J. Agric. Food Chem. 48 (2000) 5618–5623. doi:10.1021/jf0006832.
[136] F.L. Hsu, Y.C. Chen, J.T. Cheng, Caffeic acid as active principle from the fruit of Xanthium strumarium to lower plasma glucose in diabetic rats, Planta Med. 66 (2000) 228–230. doi:10.1055/s-2000-8561.
[137] A. Andrade-Cetto, H. Wiedenfeld, Hypoglycemic effect of Cecropia obtusifolia on streptozotocin diabetic rats, J. Ethnopharmacol. 78 (2001) 145–149. doi:10.1016/S0378-8741(01)00335-X.
[138] K.L. Johnston, M.N. Clifford, L.M. Morgan, Possible role for apple juice phenolic compounds in the acute modification of glucose tolerance and gastrointestinal hormone secretion in humans, J. Sci. Food Agric. 82 (2002) 1800–1805. doi:10.1002/jsfa.1264.
[139] Z. Liu, Z. Luo, C. Jia, D. Wang, D. Li, Synergistic Effects of Potentilla fruticosa L. Leaves Combined with Green Tea Polyphenols in a Variety of Oxidation Systems., J. Food Sci. 81 (2016) C1091-101. doi:10.1111/1750-3841.13292.
[140] P. Pignatelli, A. Ghiselli, B. Buchetti, R. Carnevale, F. Natella, G. German, F. Fimognari, S. Di Santo, L. Lenti, F. Violi, Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine, Atherosclerosis. 188 (2006) 77–83. doi:10.1016/j.atherosclerosis.2005.10.025.

6. Appendix

Appendix A: α – Amylase and α – Glucosidase Acarbose Dose-Response Curves

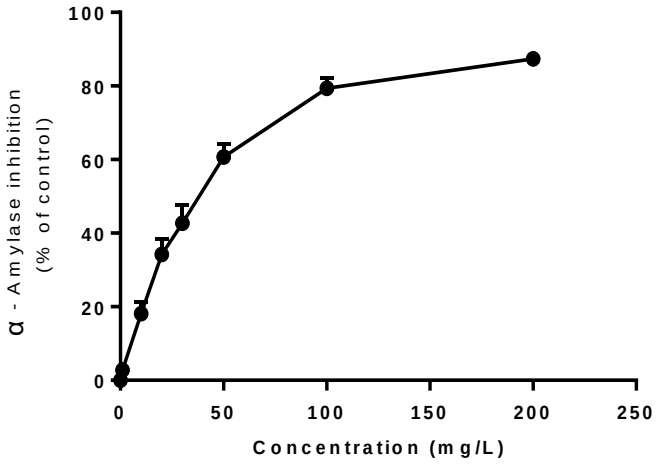

Figure 6.1: α-Amylase dose-response curve of acarbose. IC_{50} value of 35.56 ± 1.37 mg/L (≈ 55.08 µM). Results are expressed as mean ± SD of three independent experiments performed in triplicate.

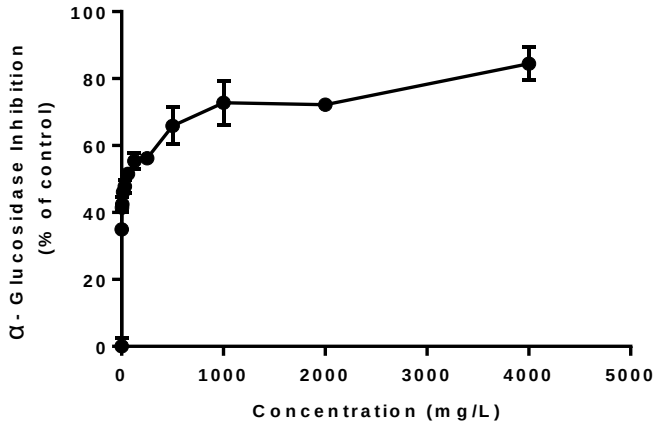

Figure 6.2: α-Glucosidase dose-response curve of acarbose. IC_{50} value of 31.9 ± 9.65 mg/L (≈ 49.4 µM). Results are expressed as mean ± SD of three independent experiments performed in triplicate.

Appendix B: α – Glucosidase CompuSyn® Synergy Analysis

Table 6.1: Combination study of the effect of acarbose and gallic acid (1:1 ratio). The analysis was done by calculation of the combination index (CI) using CompuSyn®. CI < 1, synergy; CI = 1 additive effect; CI > 1, antagonism [113].

Acarbose (mg/L)	Gallic Acid (mg/L)	Effect	CI
100.0	100.0	0.938	4.31E-6
50.0	50.0	0.8162	0.00217
25.0	25.0	0.6972	0.04423
12.5	12.5	0.5843	0.35857
6.25	6.25	0.5568	0.33789
3.13	3.13	0.5204	0.38699
1.56	1.56	0.5025	0.28902
0.78	0.78	0.4471	0.50720
0.39	0.39	0.4406	0.29425

Appendix C: Cytotoxicity of Aveleda Alvarinho (2014) and Digested Aveleda Alvarinho (2014) - 4 Hours of Incubation

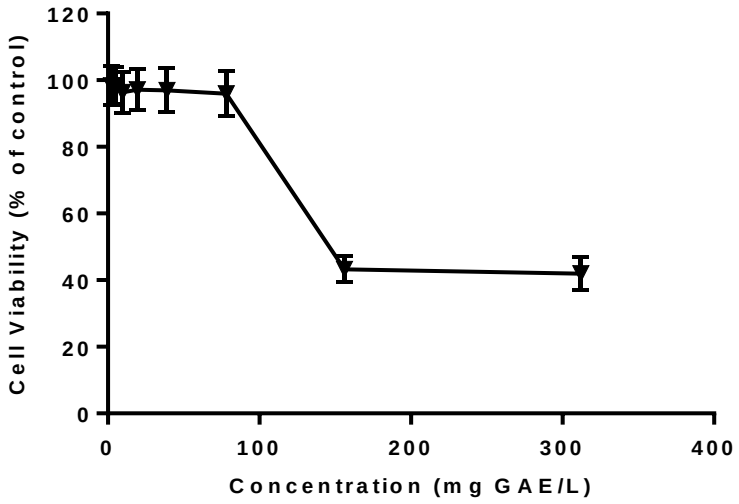

Figure 6.3: Cytotoxic evaluation of Aveleda Alvarinho (2014) on Caco-2 cells. Incubation = 4 h; Results are expressed as mean ± SD of three independent experiments performed in triplicate.

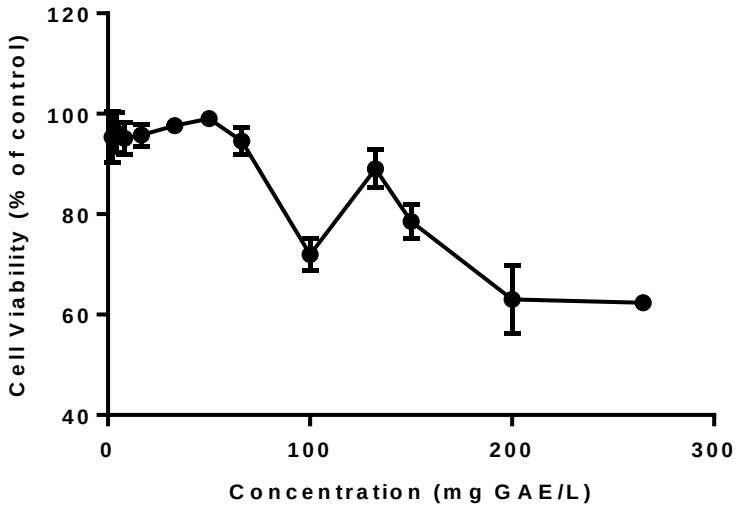

Figure 6.4: Cytotoxic evaluation of intestinal digested Aveleda Alvarinho (2014) on Caco-2 cells. Incubation = 4 h; Results are expressed as mean ± SD of three independent experiments performed in triplicate.